Investigation of mutants and substrates of the *Arabidopsis* SUMO conjugating system

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ABSTRACT

SUMOylation is a posttranslational modification of proteins that is found in the eukaryotic kingdom, but not in bacteria or archeae. During this process, SUMO, the Small Ubiquitin related modifier protein, is covalently attached onto its targets via an enzymatic cascade. SUMOylation can prevent or induce other modifications of the substrate, can lead to conformational changes and generates or abolishes binding interfaces. SUMOylation can therefore change the localization, activity, interactions or life span of a protein.

Although SUMOylation is vital in the model plant *Arabidopsis thaliana* (Saracco *et al*., 2007), only little is known about regulation and substrates of SUMO conjugation, as the temporary nature of this modification and the fact that only a small subset of substrate is modified at a given time, makes the study of SUMOylation in plants extremely difficult.

In this work, several aspects of *Arabidopsis* SUMOylation are discussed:

- An *in vitro* SUMOylation assay that utilizes plant recombinant proteins was developed. This system allows quick analysis of potential SUMOylation enzymes and substrates *in vitro*.

- The features of a SUMO1 variant, SUMO1 Q90A, in which a conserved glutamine residue at position -4 from the carboxyl terminus is changed to alanine, were analyzed *in vitro.* It was shown that this mutant variant leads to increased conjugate stability towards Early in Short Days 4 (ESD4), a major SUMO protease of *Arabidopsis*. As SUMO1 Q90A did not differ during conjugation from the wild type SUMO1 *in vitro*, this variant might be a valuable tool for future experiments to generate SUMOylated proteins, which are easier to detect and to analyze due to increased stability.

- Analysis of the potential SUMO ligases PIAS-LIKE1 (PIL1) and PIAS-LIKE2 (PIL2) indicated a slight contribution to bulk SUMO conjugation and only a minor role in flowering time regulation compared to the already well described SUMO ligase SIZ1, strengthening the importance of SIZ1 as major *Arabidops*is SUMO ligase.

- Plants with mutation in the SUMO protease ESD4 have growth defects with similarity to those of plants mutated in SIZ1. In contrast to *siz1* mutants, however, the growth defect of *esd4* mutants is not due to altered levels of the stress hormone salicylic acid. Furthermore, studies of the related SUMO protease Early in Short Days-Like1 (EL1) demonstrated that the latter enzyme does not localize to the nucleus if transiently expressed in *Nicotiana benthamiana*, and plays no obvious role in the regulation of flowering time, as *el1* mutants flower at a time similar to wild type. However, an *el1* mutation in the background of ecotype Wassilwskija might cause an altered tissue composition in the shoot.

- Analysis of the type III effector protein Factor X of the plant pathogen *Xanthomonas campestris* (in cooperation with Prof. Ulla Bonas and Robert Szczesny, University Halle) indicated no *in vitro* activity of this protein as SUMO, Rub1 or Ubiquitin protease.

The broad variety of aspects discussed in this work emphasizes the importance and complexity of *Arabidopsis* SUMOylation and indicates that the understanding of this modification in plants can only be achieved by further studies and identification of *in vivo* SUMO substrates. In future the SUMOylation assay system, developed in this work, and the described SUMO1 Q90A variant might help to accomplish these tasks.

ZUSAMMENFASSUNG

SUMOylierung ist eine post-translationale Proteinmodifikation, die bei eukaryotischen Organismen, nicht aber bei Bakterien oder Archeaen auftritt. Während dieses Prozesses wird SUMO, das Small Ubiquitin related modifier Protein, durch einen enzymatischen Zyklus kovalent an seine Zielproteine gebunden. SUMOylierung kann andere Modifikationen verhindern oder unterstützen, Konformationsänderungen hervorrufen oder Bindestellen für andere Interaktionspartner generieren oder zerstören. Folglich kann SUMOylierung die Lokalisation, Aktivität, Wechselwirkungen oder Lebenszeit des modifizierten Proteins verändern.

Obwohl SUMOylierung für die Modellpflanze *Arabidopsis thaliana* überlebensnotwendig ist (Saracco *et al*., 2007), ist bisher nur wenig über ihre Regulation und die SUMO-Substrate bekannt. Die Erforschung der SUMOylierung in Pflanzen wird dadurch extrem erschwert, dass diese Modifikation nicht permanent ist und nur ein kleiner Teil des Substrates zu einem bestimmten Zeitpunkt modifiziert wird.

In dieser Arbeit werden verschiedene Aspekte der SUMOylierung in *Arabidopsis* diskutiert:

- Ein *in vitro* SUMOylierungsassay basierend auf pflanzlichen, rekombinanten Proteinen wurde entwickelt. Diese Methode erlaubt die schnelle Analyse potentieller SUMOylierungsenzyme und Substrate *in vitro*.

- Die Eigenschaften eines mutierten SUMO1 mit Q90A Mutation, in dem ein konserviertes Glutamin an der Position -4 vom Carboxylterminus gegen Alanin ausgetauscht wurde, wurden ebenfalls *in vitro* analysiert. Es konnte gezeigt werden, dass diese mutierte Version von SUMO1 zu einer

gesteigerten Stabilität der Konjugate gegen Early in Short Days 4 (ESD4), der wichtigsten SUMO-Protease in *Arabidopsis*, führt. Da sich SUMO1 Q90A *in vitro* während der Konjugation nicht vom ursprünglichen SUMO1 unterscheidet, könnte diese Variante in zukünftigen Experimenten ein Werkzeug darstellen, um SUMOylierte Proteine zu erzeugen, die aufgrund ihrer erhöhten Stabilität leichter zu untersuchen sind.

- Die Analyse der potentiellen SUMO-Ligasen PIAS-LIKE1 (PIL1) und PIAS-LIKE2 (PIL2) impliziert sowohl einen geringen Effekt dieser Proteine auf die Häufigkeit an SUMO-Konjugaten in der Pflanze, als auch eine untergeordnete Rolle bei der Blühzeitpunktkontrolle im Vergleich zu der bereits gut beschriebenen SUMO-Ligase SIZ1, wodurch die Rolle von SIZ1 als wichtigste SUMO-Ligase in *Arabidopsis* bestätigt wird.

- Pflanzen mit einer Mutation der SUMO-Protease ESD4 haben einen ähnlichen Wachstumsdefekt wie Pflanzen mit einer Mutation von SIZ1. Im Gegensatz zu *siz1* ist dieser Wachstumsdefekt jedoch nicht auf veränderte Level des Stresshormones Salicylsäure zurückzuführen. Darüber hinaus haben Untersuchungen der ähnlichen SUMO-Protease Early in Short Days-Like1 (EL1) gezeigt, dass dieses Enzym bei transienter Expression in *Nicotiana benthamiana* nicht im Nukleus lokalisiert ist und dass es keine offensichtliche Rolle in der Regulation des Blühzeitpunktes spielt, da *el1* Mutanten zu einem ähnlichen Zeitpunkt wie der Wildtyp blühen. Allerdings scheint eine *el1* Mutation im Ökotypen-Hintergrund Wassilewskija eine veränderte Gewebezusammensetzung im Spross hervorzurufen.

- Die Analyse des Typ III-Effektors Faktor X des Pflanzenpathogens *Xanthomonas campestris* impliziert, dass dieses Protein *in vitro* weder als SUMO-, noch als Rub1- oder als Ubiquitin-Ligase aktiv ist (in Kooperation mit Prof. Ulla Bonas und Robert Szczesny, University Halle).

Die Bandbreite an Aspekten, die in dieser Arbeit besprochen wird, zeigt die Wichtigkeit und Komplexität der SUMOylierung in *Arabidopsis* auf. So wird deutlich, dass ein besseres Verständnis dieser Modifikation in Pflanzen nur durch weitere Untersuchungen und durch die Identifikation von SUMO-Substraten *in vivo* erreicht werden kann. In Zukunft könnten der *in vitro* SUMOylierungsassay, welcher in dieser Arbeit entwickelt wurde, und die beschriebene SUMO-Variante SUMO1 Q90A dabei helfen, dieses Ziel zu erreichen.

TABLE OF CONTENT

1 INTRODUCTION

During their development or as a response to biotic or abiotic influences from the environment, organisms have to control the activity of proteins. One way to adjust the abundance of proteins indirectly is the regulation of transcription, RNA processing and translation, to modify the synthesis of proteins. Another concept are post-translational modifications that alter the characteristics of already existing proteins.

Often proteins are modified via the attachment of small groups to certain residues, for example phosphate or acetyl groups. Another way to alter proteins in eukaryotic organisms, which gets more and more into focus, is the covalent attachment of small proteins.

A well-studied example for these regulatory proteins is Ubiquitin, but several protein modifiers have been found so far (Dohmen, 2004). As they usually share the typical Ubiquitin fold and are similarly attached to their substrates via an enzymatic cascade, they are named Ubiquitin-like proteins (Ubls). Different Ubls are believed to have distinct and diverse effects on their substrates.

SUMO, the Small Ubiquitin-related Modifier protein, is one of these proteins. It plays an important role during development and response to environmental factors and modifies a vast abundance of substrates. Although SUMOylation is vital for most eukaryotic organisms, only little is known about this process in the model plant *Arabidopsis thaliana*. This work focuses on the enzymes involved in *Arabidopsis* SUMOylation, potential substrates and the development of an *in vitro* SUMOylation assay, which allows the analysis of plant recombinant proteins for their modification by SUMO and the detailed study of components of the enzymatic cycle of SUMOylation.

1.1 SUMOylation

1.1.1 SUMO

SUMO was discovered independently in different studies in 1996. This explains, why it is found under many different names like “SMT3”, “Sentrin”, “Ubl1”, “PIC1” or “GMP-1”. As the most common term and the name that is used for the *Arabidopsis* proteins is “SUMO”, this notation is used in this work.

SUMO, the Small Ubiquitin-related Modifier protein, belongs to the group of the Ubls, Ubiquitin-like proteins that share the typical beta grasp (ββαββαβ) fold of Ubiquitin (Dohmen, 2004). Like all known Ubls so far it is exclusively found in eukaryotic organisms, but absent in bacteria and archeae (Meulmeester & Melchior, 2008), although there seems to be an evolutionary link to proteins involved in bacterial Molybdenum metabolism (Dohmen, 2004).

Unlike some other Ubls, SUMO has only a low sequence similarity of about 18% to Ubiquitin. It is also distinguished by a flexible N-terminal extension and differs in its surface charge. It was shown that human SUMO2 has a strongly negative region, while the corresponding area in Ubiquitin is neutral (Huang *et al*., 2004). Both protein modifiers have a di-glycine (Gly-Gly) motif at the carboxyl terminus and are attached to their substrate via a similar enzymatic cycle.

1.1.2 The SUMOylation process

Like Ubiquitin, SUMO is transferred onto its substrated via an enzymatic cascade that is similar to the different steps of Ubiquitylation.

In Figure 1.1 a scheme of the SUMOylation cycle (in *Arabidopsis thaliana*) is shown.

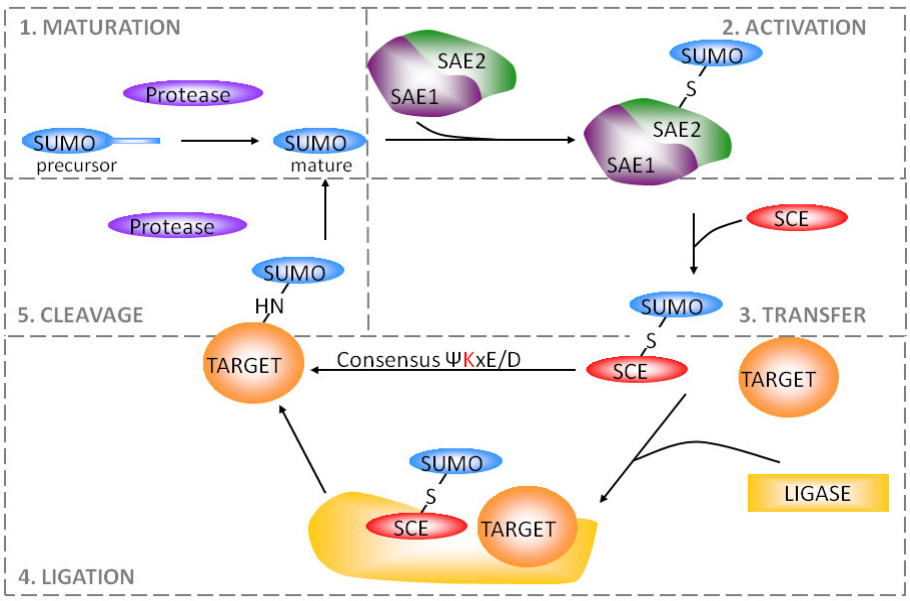

Figure 1.1: SUMOylation Cycle

SUMO, the Small Ubiquitin-like Modifier protein, is translated as a precursor that has to undergo proteolytical cleavage. Then, the mature SUMO moiety can be activated by the heterodimeric SUMO Activating Enzyme (SAE) via thioester formation. SUMO is transferred from the SAE to the SUMO Conjugating Enzyme (SCE). It can be directly attached to the target by SCE via a peptide bond to a lysine residue, if the potential substrate contains a so-called SUMOylation consensus motif (ΨKxE/D). Proteins lacking this motif can be modified nevertheless, but in this case, an additional SUMO ligase is needed to promote the reaction. SUMOylation is a reversible process, because the modifier can be cleaved off again by SUMO proteases.

SUMO is first translated as a precursor protein and has to undergo proteolytical cleavage by SUMO proteases that reveal the double glycine (Gly-Gly-) motif at the carboxyl terminus (Dohmen, 2004; Meulmeester & Melchior, 2008).

Afterwards it is activated by the SUMO Activating Enzyme (SAE) or E1. This enzyme is a heterodimer consisting of SAE1 (Aos1, Sua1) and SAE2 (Uba2) with sequence similarities to the amino and carboxyl-terminal parts of Ubiquitin activating enzymes. During the activation a thioester bound between SUMO and an active site cysteine in SAE2 is formed in an ATP-dependent process.

Then, the SUMO moiety is transferred to the SUMO Conjugating Enzyme (SCE, Ubc9) also named E2. In contrast to the different E2s involved in Ubiquitylation, only a single E2 for SUMOylation was found in different eukaryotic organisms so far.

After thioester formation with the E2, two different possibilities exist:
On the one hand, SUMO can be directly transferred onto its target proteins by the E2. This is the case, if the substrate has a so-called SUMOylation consensus sequence, a tetrapeptide consisting of the amino acids ΨKxE/D. In this motif a bulky hydrophobic amino acid, Ψ, is followed by the lysine to which the SUMO is attached, and after the spacer residue x the consensus motif ends with an acidic residue. It was shown that the E2 can bind at least a subset of substrates via this motif (Sampson *et al*., 2001). This recognition motif can be extended to the PDSM, the phosphorylation-dependent SUMOylation motif (ΨKxE/DxxSP), which contains an additional phosphorylation site in a defined distance or to the NDSM, the negatively charged amino acid-dependent SUMOylation motif that has negatively charged residues at position +3 to +6 from the consensus motif (Anckar *et al*., 2006; Hietakangas *et al*., 2006; Yang *et al*., 2006).
On the other hand, motifs with no resemblance to the consensus sequence can be SUMOylated nevertheless. In this case, an additional SUMO ligase is needed, to promote SUMOylation by bringing the SUMO-E2 conjugate and the substrate in a favorable spatial relation.

1.1.3 Mechanisms and interactions with other signaling pathways

SUMOylation can alter the fate of its target proteins by different mechanisms.
On the one hand, the SUMO moiety can block the lysine residue to which it is attached. So it can prevent other post-translational modifications like Ubiquitylation, acetylation or methylation (Meulmeester & Melchior, 2008). The SUMO protein can also block a potential binding side for another interacting protein, or on the other hand it can generate a binary binding site, which allows high-affinity interactions with another binding partner. The latter is often the case, if the interactor contains a so called SUMO Interacting Motif (SIM). This motif compromises the sequence V/I/L V/I/L x V/I/L or V/I/L x V/I/L V/I/L and is often

accompanied by a stretch of acidic amino acids. It can form a beta sheet with the second beta strand of the SUMO moiety either in parallel or anti-parallel (Hecker *et al*., 2006; Knipscheer *et al*., 2008; Meulmeester & Melchior, 2008; Perry *et al*., 2008).

These types of motifs were found in Ubiquitin ligases of different organisms, leading to their classification as SUMO targeted Ubiquitin ligases (StUbls). It was demonstrated that this group of enzymes preferably ubiquitylates substrates that were SUMOylated before, undermining the traditional idea that SUMO generally stabilizes substrates by preventing their Ubiquitiylation (Lallemand-Breitenbach *et al*., 2008; Mullen & Brill, 2008; Prudden *et al*., 2007; Tatham *et al*., 2008).

Hietakangas and others could identify the phosphorylation-dependent SUMOylation motif (PDSM), ΨKxE/DxxSP, and demonstrated that phosphorylation of a serine residue at a conserved distance can directly trigger SUMOylation of mammalian proteins, for example heat shock factors (Hietakangas *et al*., 2006). This result suggests a direct link between SUMOylation and the diverse signaling pathways via MAP kinases.

It is also possible that SUMOylation leads to an intramolecular conformational change if the target protein contains not only a SUMOylation site but also a SUMO interaction motif (Meulmeester & Melchior, 2008). Figure 1.2 shows an overview of the working mechanisms underlying SUMOylation.

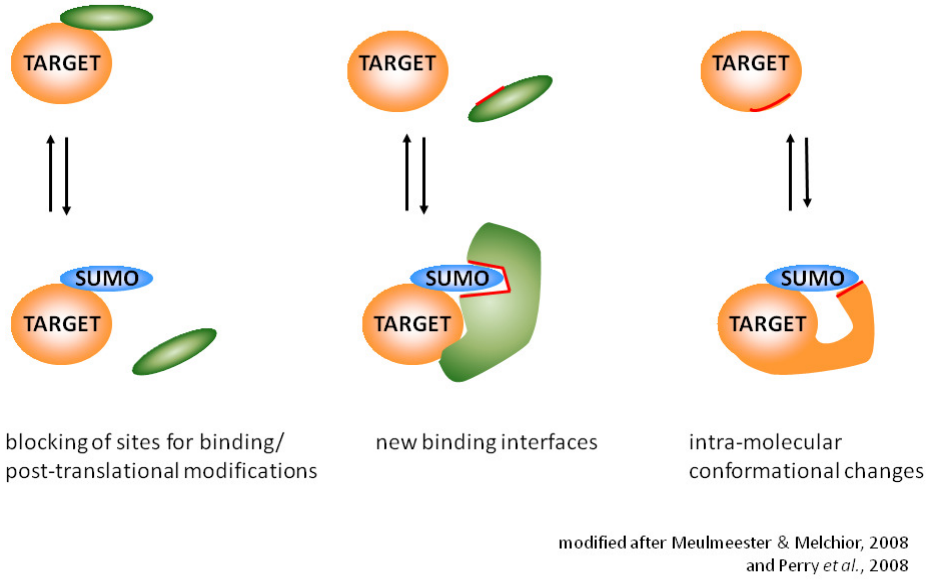

Figure 1.2: Mechanisms of SUMOylation

The attachment of a SUMO moiety to a target protein can have several effects. On the one hand, it can block a binding site of other proteins (depicted in green) or prevent other modifications of the lysine residue to which it is attached. On the other hand, SUMOylation can create new binding interfaces or leads to intermolecular changes, if the interactor or the substrate itself contains a SUMO interaction motif symbolized by a red line.

1.2 SUMOylation in different eukaryotic organisms

As mentioned before, the attachment of SUMO will alter the properties of a protein and might even prevent other modifications or protein–protein interactions. In this chapter some general effects of SUMOylation, observed in different organisms as yeast or mammals, are shown. Detailed information about SUMOylation in the model plant *Arabidopsis thaliana* are given in the next chapter.

SUMOylated proteins are often found in the nucleus and SUMO can regulate their localization. For example, SUMOylation plays a role in the import of proteins into the nucleus (Dohmen, 2004). The GTPase-activating protein RanGAP that is involved in nuclear ex- and import was the first identified SUMO target. RanGAP

is cytoplasmic in its non-SUMOylated state, but if SUMOylated, it interacts with RanBP2, a SUMO ligase, which is part of the Nuclear Pore Complex (NPC). It was also reported that for some proteins like NEMO, which is involved in signal transduction after genotoxic stress, SUMOylation is sufficient to facilitate nuclear localization (Huang *et al*., 2003). Not only import into the nucleus can be dependent on SUMOylation, but also subnuclear localization is regulated by SUMO. In case of so-called PML nuclear bodies, which are characterized by the tumor suppressor promyelocytic leukemia protein (PML), SUMOylation of PML is vital for formation of these compartments (Duprez *et al*., 1999; Shen *et al*., 2006). Many other proteins are found in PML bodies only in their SUMOylated state (Gill, 2004; Janssen *et al*., 2007).

As SUMOylated proteins are often found in the nucleus, it is not surprising that many processes in this organelle are regulated by SUMOylation. A major effect of SUMOylation is transcriptional repression. Many transcription factors can be inhibited by SUMO modification, because SUMOylation can trigger interaction with co-repressors (Gill, 2005). It was also proposed that SUMO regulates chromatin structures by targeting several histone deacetylases such as HDAC1 or HDAC4, resulting in increased transcriptional repression by these proteins (David *et al*., 2002). SUMOylation of histone H4 also mediates silencing by attracting histone deacetylases (Shiio & Eisenman, 2003).

Another process regulated by SUMO is cell division. Studies in yeast and mammalian systems revealed that SUMOylation functions in cell cycle control by modifying distinct proteins only at certain phases of the cell cycle. Therefore, a disturbance of the SUMO cycle can cause problems in progression of the cell cycle and chromosome segregation (Dieckhoff *et al*., 2004; Hayashi *et al*., 2002; Makhnevych *et al*., 2007; Nacerddine *et al*., 2005).

SUMO influences also DNA damage repair. In budding yeast, the Proliferating Cell Nuclear Antigen (PCNA) is SUMOylated at its lysine residue K164, and by

this modification Ubiquitin-dependent DNA damage tolerance is inhibited, as both Ubiquitin and SUMO compete for attachment to this residue (Ulrich, 2004; Ulrich, 2007).

SUMO is also involved in the signaling response to different environmental factors. In different organisms an increase of SUMO conjugates was observed after stress treatment such as heat shock (Anckar *et al*., 2006; Hietakangas *et al*., 2006; Kurepa *et al*., 2003; Saitoh & Hinchey, 2000) and many proteins involved in signal transduction are SUMOylated in mammalian cells (Dohmen, 2004; Gao & Karin, 2005; Gill, 2004; Huang *et al*., 2003).

Even mitochondrial fission (in mammals) seems to be regulated via SUMOylation of the Dynamin Related Protein 1 (DRP1), although these organelles are of bacterial origin (Harder *et al*., 2004; Zunino *et al*., 2007).

Links to different diseases are also found for SUMOylation. On the one hand, SUMOylation plays an important role in diseases such as cancer that are caused by an aberrant cell cycle, and in (often neuro-degenerative) diseases such as Alzheimer´s disease or Huntington, which are caused by increased stability and accumulation of certain proteins (Dorval & Fraser, 2007; Kim & Baek, 2006; Lallemand-Breitenbach *et al*., 2008; Mo & Moschos, 2005; Steffan *et al*., 2004; Zhang & Sarge, 2008).

Some pathogens, for example herpes virus, also inject proteins into host cells, which can either be SUMOylated by the host SUMO system or that manipulate SUMOylation of the host cell to promote the infection (Adamson & Kenney, 2001; Bailey & O'Hare, 2002; Boggio & Chiocca, 2006; Kang *et al*., 2006; Lee *et al*., 2004; Muller & Dejean, 1999; Orth *et al*., 2000).

1.3 The *Arabidopsis thaliana* SUMO conjugating system

The *Arabidopsis* SUMO conjugating system is distinct from those of other organisms by its broad abundance of different SUMO isoforms. Baker´s yeast has only a single SUMO gene, smt3, and four SUMO isoforms were found in humans so far. The *Arabidopsis* genome codes for eight different SUMO isoforms, of which at least four are expressed to a significant extent (Novatchkova *et al.*, 2004). Below an alignment of the SUMO variants is shown.

```
SUMO1     ............MSANQEEDKKPGDGGAHINLKVKGQDGNEV.FFRIKRSTQLKKLM
SUMO2     ............MSATPEEDKKP.DQGAHINLKVKGQDGNEV.FFRIKRSTQLKKLM
SUMO3     ............MSNPQDDKPIDQEQEAHVILKVKSQDGDEV.LFKNKKSAPLKKLM
SUMO5     ...MVSSTDTISASFVSKKSRSPETSPHMKVTLKVKNQQGAED.LYKIGTHAHLKKLM
SUMO4     MST..TSRVGSNEVKMEGQKRKVV.SDPTHVTLKVKGQDEEDFRVFWVRRNAKLLKMM
SUMO6     MSTKSSSIHGRNEVKMEGEKRKDVESESTHVTLNVKGQDEEGVKVFRVRRKARLLKLM
SUMO7     ................MSAADKKPLIPPSHITIKIKSQDDICV.YFRIKRDVELRTMM
SUMO7v    ................MSAADKKPLIPPSHITIKIKSQDDICV.YFRIKRDVELRTMM
SUMO8     ................MSSSDKKPLIPSSHITVKVKNQDDICV.YFRIKRDVELRKMM

SUMO1    NAYCDRQSVDMNSIAFLF.DGRRLRAEQTPDE...LDMEDGDEIDAMLHQTGG/SGGGATA
SUMO2    NAYCDRQSVDFNSIAFLF.DGRRLRAEQTPDE...LEMEDGDEIDAMLHQTGG/GAKNGLKLFCF
SUMO3    YVYCDRRGLKLDAFAFIF.NGARIGGLETPDE...LDMEDGDVIDACRAMSGG/LRANQRQWSYMLFDHNGL
SUMO5    SAYCTKRNLDYSSVRFVY.NGREIKARQTPAQ...LHMEEEDEICMVMELGGG/GPYTP
SUMO4    ELYTKMRGIEWNTFRFLF.DGSRIREYHTPDE...LERKDGDEIDAMLCQQSG/FGPSSIKFRV
SUMO6    EYYAKMRGIEWNTFRFLSDDGSRIREYHTADD...MELKDGDQIDALLPQESG/FGPSTVFRV
SUMO7    QAYSDKVGQQMSAFRFHC.DGIRIKPNQTPNE...LDLEDGDEIDAFVDQIAG/FSHRH
SUMO7v   QAYSDKVGQQMSAFRFHC.DGIRIKPNQTPNEELQLDLEDGDEIDAFVDQIAG/FSHRH
SUMO8    HAYSDKVGVEMSTLRFLF.DGNRIKLNQTPNE...LGLEDEDEIEAFGEQLGG/FSFFHRH
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Figure 1.3: Different SUMO isoforms of *Arabidopsis thaliana*

The shown alignment of the different SUMO isoforms in *Arabidopsis* was modified after Novatchkova *et al.*, 2004.
Amino acids, which are generally conserved in the SUMO core, are highlighted in yellow. Additional residues that are absent in other SUMO variants are shown in grey. Differences from the conserved glutamine residue at position -4 from the carboxyl terminus are depicted in red and aberrations from the conserved di-glycine motif in blue. The SUMOylation consensus site within SUMO2 is highlighted in green (Colby *et al.*, 2006). A cDNA splicing variant of SUMO7 is listed as SUMO7v. The cleavage side for SUMO proteases, which reveals the di-glycine motif during maturation, is indicated by a slash.

As depicted above in Figure 1.3, the *Arabidopsis* genome encodes eight potential SUMO isoforms and a ninth pseudo gene that encodes no complete protein and is therefore not shown. The isoforms SUMO1 (At4g26840) and SUMO2 (At5g55160), SUMO4 (At5g48710) and SUMO6 (At5g48700), SUMO7 (At5g55855) and SUMO8 (At5g5585x) are closely related and are probably derived from genome duplication and rearrangement events (Novatchkova *et al*., 2004). SUMO8 lies between At5g55855 and Atg55860 and overlaps with SUMO7 (Novatchkova *et al*., 2005).

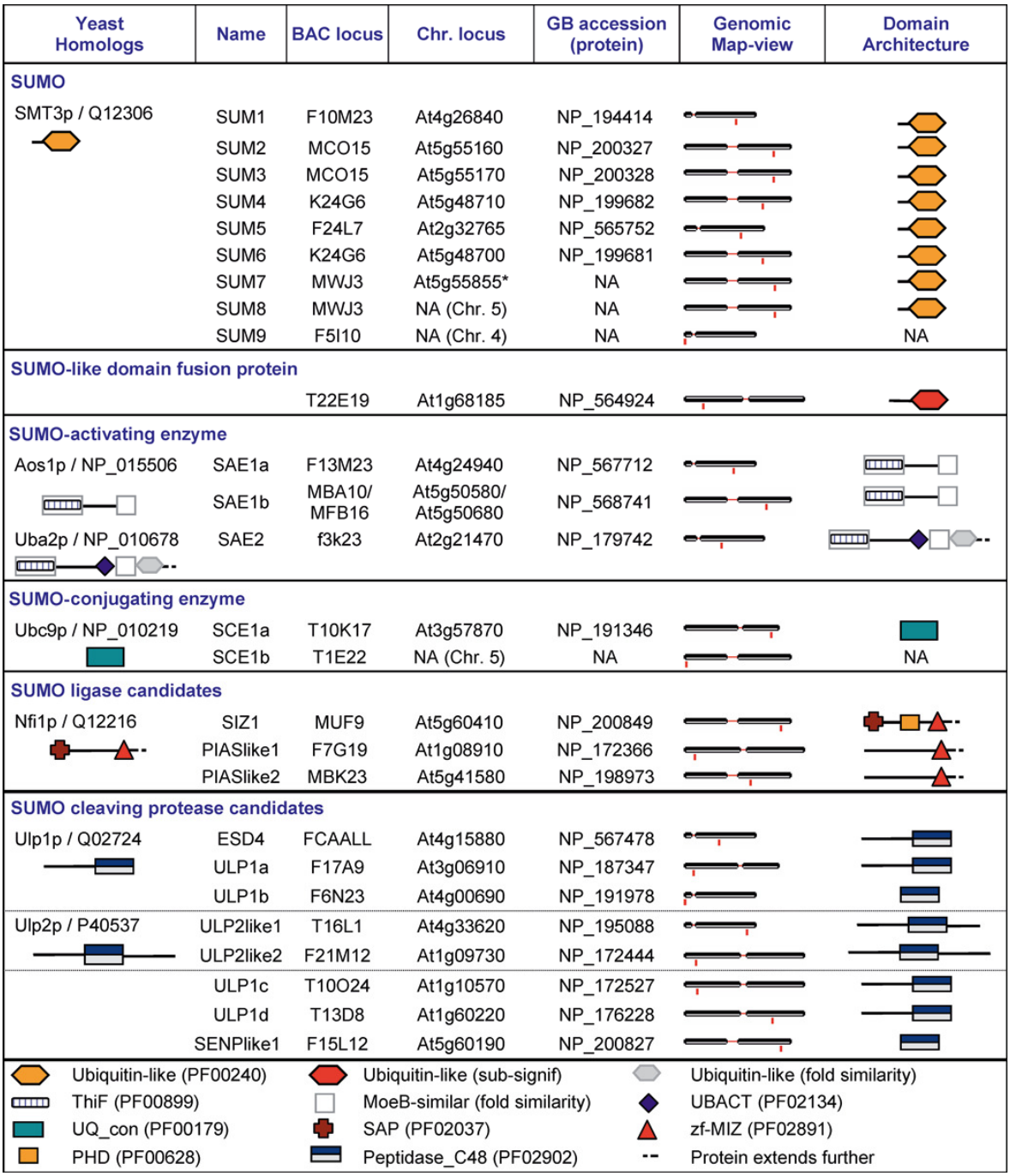
It seems that only the isoforms SUMO1, 2, 3 and 5 are expressed to a significant extent and therefore this work is restricted to those variants (Kurepa *et al*., 2003; Saracco *et al*., 2007).

The isoforms SUMO1 and SUMO2 show the highest resemblance to human SUMO2 and SUMO3. Like the mammalian isoforms, they form conjugates *in vivo* and after stress treatment the level of those conjugates increases drastically (Kurepa *et al*., 2003). Thomas Colby and co-worker demonstrated chain formation of SUMO2, during which a lysine residue close to the carboxyl terminus that is part of a consensus motif is modified (Colby *et al*., 2006).

Only little is known about SUMO3 and SUMO5. Both variants seem to form conjugates *in vivo*, but the conjugate level is not influenced by stress treatment such as heat shock (Budhiraja, 2005; Kurepa *et al*., 2003). Due to the lack of a conserved glutamine residue at position -4 of the amino terminus, the conjugate stability of SUMO3 and SUMO5 might be increased, because this amino acid is part of a recognition site for SUMO proteases (Mossessova & Lima, 2000; Reverter & Lima, 2004; this work).

Novatchkova and co-workers identified not only the different SUMO isoforms but also a broad variety of potential SUMOylation enzymes in *Arabidopsis* with homology to the respective yeast orthologs as shown in Table 1.1.

Table 1.1: Components of the *Arabidopsis* SUMO conjugating system



This figure was taken from Novatchkova *et al.*, 2004. Potential orthologs of *Saccharomyces cerevisae* and *Arabidopsis thaliana* were identified. SUMO is abbreviated as SUM.

As depicted above in Table 1.1, *Arabidopsis* encodes two different isoforms of the subunit 1 of the SUMO activating enzyme. Either SAE1a (At4g24940) or SAE1b (At5g50680) can form a heterodimer with SAE2 (At2g21470) to generate the functional enzyme. Only a single gene coding for the SUMO conjugating enzyme

is found in *Arabidopsis*. In this, SUMOylation differs clearly from Ubiquitylation as many different conjugating enzymes for Ubiquitin are known (Bachmair *et al*., 2001; Dohmen, 2004). Three potential SUMO ligases exist in *Arabidopsis*, but only one of them, the SIZ1 (At5g60410) has already been described and its biological relevance was demonstrated. The other two members of the SIZ/PIAS protease family in *Arabidopsis*, PIAS-Like1 (At1g08190) and PIAS-Like2 (At5g41580) will be discussed in this work.

The *Arabidopsis* genome codes for several potential SUMO proteases, but Colby and others demonstrated that SENPLike1 (At5g60190) is more likely processing Rub1, another member of the Ubl family (Colby *et al*., 2006). *In vitr*o SUMO protease activity for Ulp1d (At1g60220), Ulp1c (At1g10570), ESD4 (At4g15880) and Ulp1a, also named Early in short days 4-Like 1 (EL1, At3g06910) was demonstrated (Colby *et al*., 2006; Murtas *et al*., 2003), but only the influence of Early in short days 4 (ESD4) has been analyzed *in planta* as described in the next chapter. EL1, the closest homolog of ESD4 in *Arabidopsis*, will be further investigated in this work.

1.4 The role of SUMO in *Arabidopsis thaliana*

Richard Vierstra and his co-workers demonstrated the vital role of SUMOylation in *Arabidopsis.* They showed that disruption of the SUMO cycle caused either by the double knockout of the nearly identical isoforms SUMO1 and SUMO2 or by loss of function mutations in SCE or SAE2, which are both encoded by single genes, are lethal during early plant embryogenesis (Saracco *et al*., 2007).

SUMOylation is involved in many different developmental processes and responses to biotic and abiotic factors in plants. Most of these insights were obtained by studying mutants in different SUMOylation enzymes.

It was shown that the amount of SUMO1 and SUMO2 conjugates increases in *Arabidopsis* after stress treatments, for example heat shock (Kurepa *et al*., 2003). This result parallels the increased occurrence of SUMO conjugates in other organisms after stress and is strengthened by the result that plants, lacking SIZ1, a SUMO protease of the PIAS family, show reduced tolerance to drought, cold stress and phosphate starvation (Catala *et al*., 2007; Miura *et al*., 2007; Miura *et al*., 2005; Yoo *et al*., 2006).

SUMOylation plays also a role in plant hormone signaling. It was demonstrated that *siz1* mutant plants have elevated levels of salicylic acid (Jin *et al*., 2008b; Lee *et al*., 2007). Recent findings suggest that the reduced growth of *siz1* plants is partly due to decreased expression of genes involved in brassinosteroid synthesis in signaling (Catala *et al*., 2007). SIZ1 does not seem to be involved in jasmonic acid signaling (Lee *et al*., 2007). However, SUMOylation seems to modify absicisic acid (ABA) signaling in plants. It was shown that overexpression of SUMO1 and SUMO2 in *Arabidopsis* attenuates ABA-induced growth arrest and leads to a stronger induction of ABA- and stress-responsive genes, while reduction of the SCE expression has an opposite effect (Lois *et al*., 2003). Paul Hasegawa and his co-workers recently suggested a role for SIZ1-mediated SUMOylation in the regulation of auxin signaling and transport during phosphate starvation, but up to now this theory is lacking full experimental proof (Miura *et al*., 2007; Miura *et al*., 2005). The SUMOylation system is also involved in plant-pathogen interactions. *Siz1* mutant plants show constitutive systemic acquired resistance due to their elevated salicylic acid levels and constitutive expression of genes involved in pathogenesis with the effect of increased resistance to *Pseudomonas syringae* (Lee *et al*., 2007). The pathogenic bacterium *Xanthomonas campestris*, for example, secretes the virulence factors AvrXv4 and XopD into host cells, which show SUMO protease activity (Chosed *et al*., 2007; Colby *et al*., 2006; Gurlebeck *et al*., 2006; Hotson *et al*., 2003; Roden *et al*., 2004). The bacterial protein XopD seems to be highly specialized in the cleavage of plant proteins, because it was demonstrated

that this enzyme can process efficiently tomato and *Arabidopsis* SUMO precursors and conjugates but not mammalian and yeast SUMO isoforms (Chosed *et al*., 2007; Colby *et al*., 2006; Hotson *et al*., 2003). These results lead to the idea that plant pathogens use proteins with SUMO protease activity to perturb the host´s SUMOylation system.

SUMOylation has also a function in the control of flowering time. Mutants of the SUMO ligase SIZ1 or of the SUMO protease ESD4 both flower early under short day conditions (Jin *et al*., 2008; Murtas *et al*., 2003). SUMO protease activity for ESD4 was demonstrated *in vitro* and this protein seems to regulate the abundance of Flowering Locus C (FLC), a floral repressor (Colby *et al*., 2006; Murtas *et al*., 2003; Reeves *et al*., 2002). The early flowering of *siz1* mutant plants might be caused by their altered levels of salicylic acid, or by a lower level of FLC, which is repressed by the floral promoter Flowering Locus D (FLD) (Jin *et al*., 2008; Lee *et al*., 2007). SIZ1 seems to negatively control flowering time by inactivating FLD through SUMOylation (Jin *et al*., 2008).

2 RESULTS

In this work, several aspects of *Arabidopsis* SUMOylation are discussed. Therefore, this chapter addresses the following subjects: the development of an *in vitro* SUMOylation assay system, the mutant variety SUMO1 Q90A, different SUMO ligase and protease mutants and a potential bacterial SUMO protease. Nonetheless all projects aim hand in hand to achieve a better understanding of the plant SUMO system and benefit from each other.

2.1 *In vitro* SUMOylation

Due to the transient nature of modification by SUMO and to the small subset of proteins modified at a given time, SUMOylation is extremely hard to detect *in vivo*. Therefore we developed a SUMOylation assay system that allows the quick analysis of a broad variety of proteins for their SUMOylation by different SUMO isoforms *in vitro*. The assay is based on a system described by Prof. Frauke Melchior but the novelty is that it is entirely based on and adapted to plant recombinant proteins (Bossis & Melchior, 2006; Pichler *et al*., 2005).

2.1.1 *In vitro* SUMOylation assays – a handy technique

For *in vitro* SUMOylation assays, the necessary enzymes, SUMO moieties and potential target proteins were produced as plant recombinant proteins in *E. coli*.
The SUMO activating enzyme (SAE) consists of two subunits, SAE1, for which two isoforms (SAE1a and SAE1b) exist in *Arabidopsis*, and SAE2 that is encoded by only a single gene and contains the catalytically active cysteine. Previous results indicated that the SAE2 subunit is not very active if singly expressed in *E. coli*. This

heterodimeric enzyme was therefore expressed in *E. coli* using a dicistronic construct in which the SAE2 subunit is expressed as first reading frame, followed by either the SAE1a or the SAE1b subunit with an amino terminal hexa-histidine tag. This construct resembles poly-cistronic mRNAs of *E. coli* that encode ribosomal proteins (Yates & Nomura, 1980). It was assumed that this avoids ribosome disassembly after translation of the first protein, allowing nearly equimolar production of the two SAE subunits. To strengthen their interaction, ATP was added to a final concentration of 1 mM during protein purification.

The SUMO conjugating enzyme (SCE) was also purified as an untagged protein, because it was not possible to maintain this protein in a stably active form with either carboxyl or amino terminal His tag (data not shown).

In contrast to SCE, the other proteins were produced as His tag fusions to allow cheap and easy purification. The potential substrates and the SUMO moieties carried additional tags to allow their detection and distinction by Western blot.

For a typical *in vitro* SUMOylation assay 100 µg SUMO, 4 µg SAE, 0.6 µg SCE and 0.15 µg SIZ1 fragment were incubated with usually 10 µg substrate in an ATP and $MgCl_2$ containing buffer at 30°C for four hours or overnight. Afterwards 10 µl of the reaction were separated via SDS-PAGE and analyzed by Western blot.

It might be difficult to purify active recombinant plant SCE, but as shown in Figure 2.1, SUMOylation with SUMO3 can be more efficient with *Arabidopsis* enzymes than with commercially available human enzymes.

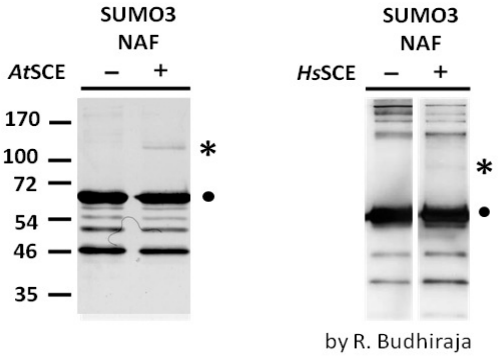


Figure 2.1: Higher efficiency of *Arabidopsis* recombinant SCE

Recombinant model substrate NAF, Nucleosome Assembly Factor, carrying a Flag tag, was incubated with recombinant SAE, and SUMO1 in ATP containing buffer. In the positive samples (+) active SCE was added, in the negative controls (-) the mutant SCE (C94S) was used that is no longer catalytically active. The results for human recombinant *Hs*SCE were obtained by Dr. Ruchika Budhiraja. Higher molecular weight bands, indicating SUMOylation, are highlighted by an asterisk while unmodified substrate is marked with a black dot.

Above, the *in vitro* SUMOylation of the model substrate NAF, a Nucleosome Assembly Factor, with SUMO3 was monitored.

Therefore recombinant enzymes, SUMO moieties and the Flag-NAF construct were incubated in an ATP containing buffer, separated by SDS-PAGE and analyzed by Western blot with anti-Flag antibody. Always a positive sample with active SCE was compared to a negative control, in which inactive SCE(C94S) was used. A higher molecular weight band, marked by an asterisk, indicates mono-SUMOylation of NAF, because the mobility on the gel corresponds to the combined mass of the NAF protein and a recombinant SUMO moiety. In this experiment, SUMOylation of NAF with SUMO3 is far stronger, if plant recombinant SCE is used in comparison to *Hs*SCE.

For the SUMO isoform SUMO1 such a difference was not detected. In some cases later on human recombinant E2 and E1 (BostonBiochem) were used, because it proved to be difficult to get the SAE and SCE as recombinant plant proteins reliably active.

To monitor the differences between the two isoforms of SAE1, SAE1a and SAE1b, and to access the influence of a functional fragment of the SUMO ligase SIZ1, their effect on chain formation of different SUMO isoforms was investigated (Figure 2.2).

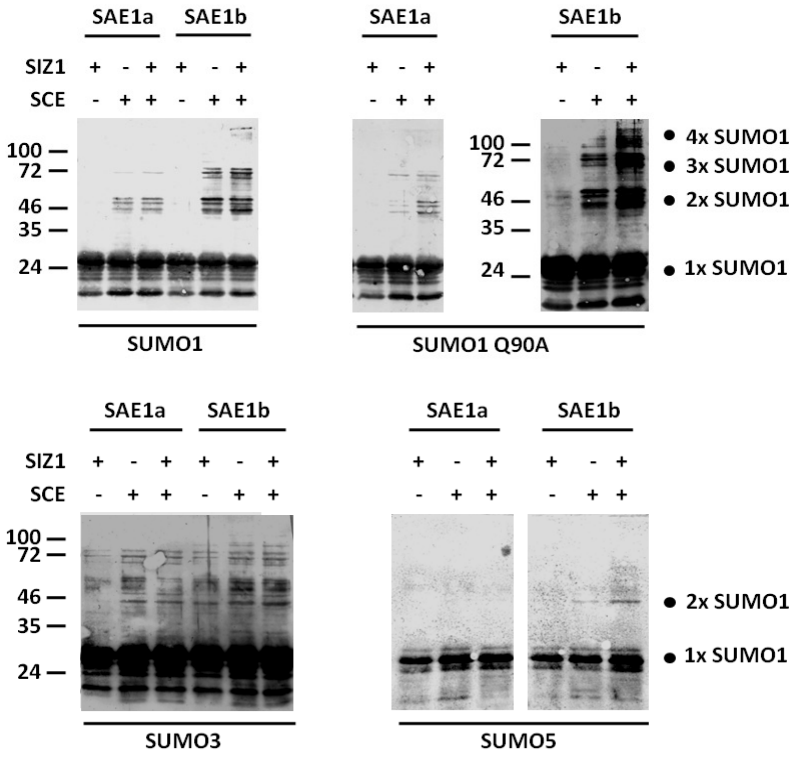


Figure 2.2: Effects of different SAE isoforms and a SIZ1 fragment on SUMO chain formation

SUMO1 moieties carrying an HA tag were incubated with recombinant SAE1a or SAE1b, SCE and a functional fragment of SIZ1 as indicated. An aliquot was separated by SDS PAGE and SUMO moieties were detected by anti-HA antibody.

As shown in Figure 2.2, the ability of different SUMO isoforms for chain formation was tested. Therefore recombinant SUMO moieties carrying an HA tag were incubated with the necessary enzymes in an ATP containing buffer. An aliquot was separated by SDS-PAGE and the SUMO moieties were detected by Western blot with antibodies targeting the HA epitope.

SUMO1 and the nearly identical SUMO1 Q90A, in which a glutamine residue close to the C-terminus is exchanged, form *in vitro* poly-SUMO chains with an identical pattern. The efficiency of chain formation by the two SUMO variants

differs. However, in the shown experiment, more SUMO1 Q90A was used, so that the bands cannot be compared quantitavely.

In case of the isoforms SUMO3 and SUMO5, the formation of slower migrating bands is far weaker and only one higher molecular weight band, presumably di-SUMO is prominently detected. Chain formation was increased, if the SAE1b isoform of the SUMO activating enzyme instead of SAE1a was used. The reaction could also be increased by addition of a functional fragment of the SUMO ligase SIZ1. SAE1b and the SIZ1 fragment were therefore used in subsequent experiments.

To use tagged fusion proteins in subsequent *in vitro* SUMOylation experiments, it was necessary to determine which tags cannot be SUMOylated themselves to avoid false positive results later on. Figure 2.3 shows the assessment of different tag constructs.

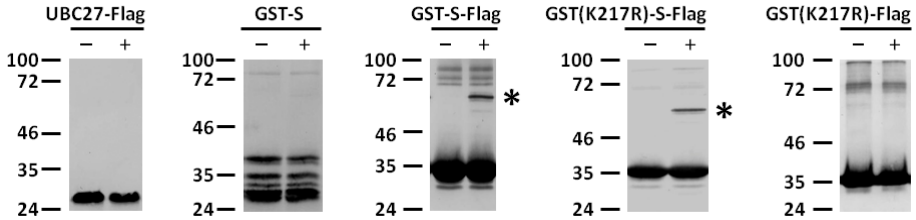


Figure 2.3: SUMOylation of different tag constructs

Different recombinant tag constructs were incubated with recombinant SAE and SUMO1 in ATP containing buffer. As the Flag peptide is too small to be expressed and purified from *E. coli*, this tag was generated as a fusion to the protein UBC27. In the positive samples (+) active SCE was added, in the negative controls the mutant SCE(C94S) was used that is no longer catalytically active. An aliquot was separated by SDS-PAGE and the constructs were detected by Western blot with either anti-Flag or anti-GST antibodies. Higher molecular weight bands, indicating SUMOylation, are highlighted by asterisks.

As shown in Figure 2.3, commonly used tags were analyzed for their *in vitro* modification by SUMO1 to establish which types of fusion proteins can be used in subsequent experiments. Only the modification by SUMO1 was tested, because this isoform was far better transferred onto target proteins during *in vitro*

experiments and to a larger extend than SUMO3 and SUMO5. As the Flag peptide is too small to be purified from *E. coli* cells, it was produced as a fusion protein with UBC27, a protein that was not modified by any tested SUMO isoform.

To test the different tags for their potential *in vitro* SUMOylation, they were incubated with the necessary enzymes and recombinant SUMO moieties and afterwards analyzed by Western blot with antibodies against the tested tag. As shown above, neither the UBC27-Flag construct, nor the combination of GST moiety and S peptide (GST-S) are SUMOylated. Still, if all three tags are combined in the protein GST-S-Flag, a higher molecular weight band occurs in the positive sample, indicating mono-SUMOylation of this construct. To determine the residue within this construct to which SUMO is attached, a lysine residue at the carboxyl terminus of the GST moiety that is in a context with similarity to a SUMOylation consensus motif, was exchanged to arginine to generate the GST(K217R)-S-Flag protein. Still, this protein did not differ in its SUMOylation from the unaltered GST-S-Flag. Therefore it was assumed that a lysine residue within the S peptide that is part of a high probability SUMOylation site might be modified and the protein GST-Flag was created. GST-Flag was no longer a substrate for SUMO1 and therefore the GST moiety and the Flag peptide have been used to create fusion proteins for subsequent experiments.

2.1.2 *In vitro* SUMOylation of different substrates

We tried to demonstrate the SUMOylation of various substrates by different SUMO isoforms. Here, we focused on SUMO1, SUMO3 and SUMO5, because these variants are expressed to a significant extent. The role of SUMO2 was not further investigated, although it is expressed to a similar extent as SUMO1, because the two isoforms are highly similar and their functional redundancy was recently demonstrated (Saracco *et al*., 2007).

A former member of our group, Dr. Ruchika Budhiraja, identified potential *in vivo* substrates of SUMO3 and 5 and a mutant version of SUMO1, the SUMO1 Q90A, from *Arabidopsis*. SUMO1 Q90A is identical to SUMO1 except for a one amino acid exchange at position -4 from the C-terminus, where the conserved Glutamine residue is changed to alanine.

Dr. Budhiraja used different transgenic plant lines expressing tagged SUMO moieties and purified SUMO conjugates via Ni-NTA affinity chromatography. After separation by SDS-PAGE, SUMO conjugates were identified by the mass spectrometric facility of the MPIZ and by Stephan Müller from the University of Cologne.

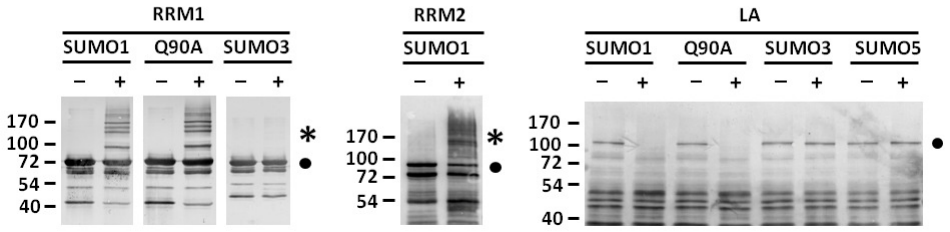
To verify the SUMOylation of the detected potential *in vivo* targets, their modification with different SUMO isoforms was analyzed *in vitro*.

Figure 2.4 shows the *in vitro* SUMOylation assays for a subset of the identified *in vivo* targets and of other proteins of interest. For a better overview, the results for the various proteins are summarized in Table 2.1.

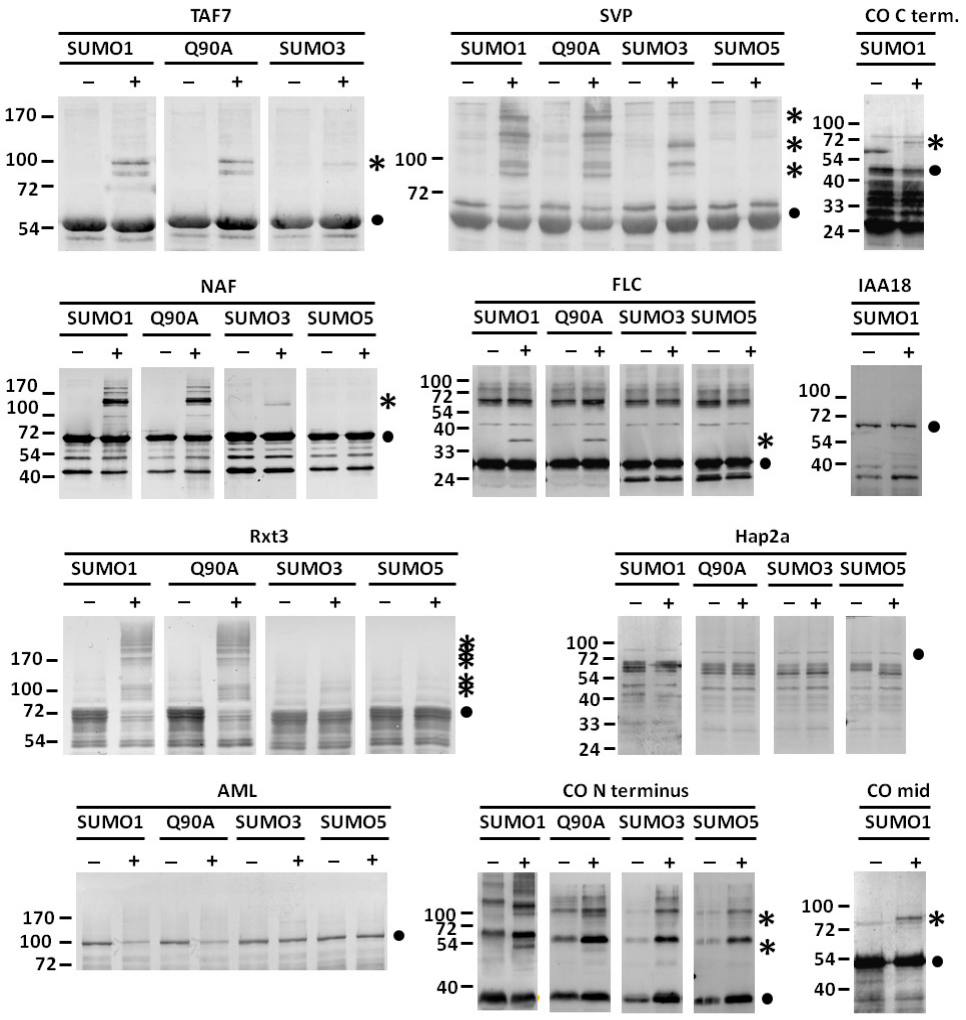
Figure 2.4: *In vitro* SUMOylation of different proteins

Various recombinant tag fusion proteins, carrying either a Flag or a GST epitope and a His tag for purification, were analyzed in an *in vitro* SUMOylation assay. The substrate was incubated with the required enzymes and different SUMO isoforms and an aliquot was separated by SDS PAGE.
Candidate proteins were detected by Western blot using anti-Flag or anti-GST antibodies. The unmodified substrate is marked with a black dot, while higher molecular weight bands, indicating SUMOylated proteins, are highlighted with asterisks.

A: RNA dependent processes



B: Chromatin and DNA related pathways



C: Others

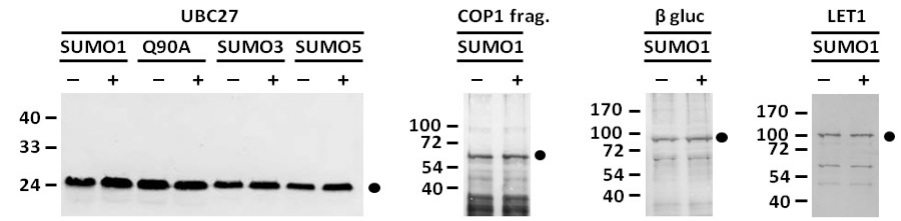


Table 2.1: ***In vitro*** **SUMOylation of different proteins**

Gene	Protein	ΨKxE/D		SIM	*in vivo* modified by SUMO			*in vitro* modified by SUMO			
		score			1	3	5	1		3	5
		≥ 0.90	≥ 0.65		Q90A			WT	Q90A		
RNA dependent processes											
At2g41060	RRM2	0	2	2	yes			yes	nt	nt	nt
At3g56860	RRM1	1	0	1	not distinguished from RRM2			yes	yes	no	nt
At2g43970	LA	0	1	1	yes			?	?	no	no
DNA and Chromatin related pathways											
At1g55300	TAF7	0	0	7	yes	yes	yes	yes	yes	yes	nt
At1g29400	AML5	0	1	0		yes		?	?	no	no
At5g08450	Rxt3	3	7	11		yes		yes	yes	no	no
At2g19480	NAF	1	1	2	yes			yes	yes	yes	no
At1g51950	IAA 18	0	4	3				no	nt	nt	nt
At5g15840	CO	2	2	3				yes	yes	no	no
At5g12840	Hap2a	0	1	0				no	no	no	no
At2g22540	SVP	0	0	3				yes	yes	yes	no
At5g10140	FLC	1	0	7				yes	yes	no	no
Other proteins											
At2g32950	COP1	2	0	3	yes			no	nt	nt	nt
At5g50870	Ubc27	0	0	1				no	no	no	no
At3g15355	PFU1	0	1	6				no	nt	nt	nt
At1g75940	β glucosidase	0	1	4	yes	yes	yes	no	nt	nt	nt

The SUMOylation of various substrates is shown. The data for the in *vivo* modification were obtained by Dr. Ruchika Budhiraja, while the *in vitro* SUMOylation resulted from this work. Unclear results for the *in vitro* modification are indicated by a question mark (?) and not tested (nt) combinations are shown as well.

As shown in Figure 2.4 and Table 2.1, the *in vitro* SUMOylation of various proteins was analyzed.

In general, it can be observed that often proteins involved in RNA dependent processes or in DNA and chromatin related pathways are targets for both *in vivo* and *in vitro* SUMOylation.

Usually, *in vitro* SUMOylation led to clear distinct higher molecular weight bands, but in case of the protein Rxt3, which contains several potential SUMOylation sites, many different higher molecular weight bands occurred.

Most proteins could be modified *in vitro* by SUMO1 and SUMO Q90A, while SUMOylation with SUMO3 was only observed for the proteins NAF, SVP and TAF7 and resulted in weaker bands compared to the other two isoforms. In contrast to SUMOylation by SUMO1 or SUMO1 Q90A, only a single slower migrating band was detected, indicating mono-SUMOylation.

SUMO5 modification was not observed at all during *in vitro* experiments, although Dr. Budhiraja had identified some potential substrates for this isoform in *Arabidopsis*. SUMO1 and SUMO Q90A are two nearly identical proteins, which differ only in one amino acid at position -4 from the carboxyl terminus. A substrate that could be modified with one variety could also be SUMOylated by the other one with an identical pattern.

For some substrates, the *in vitro* results differed from the *in vivo* prognosis. The Rtx protein for example, an *in vivo* candidate for SUMOylation with SUMO3, could not be modified with SUMO 3, but with SUMO1 and SUMO1 Q90A during *in vitro* experiments. In contrast to this, the Nucleosome Assembly Factor NAF was supposed to be only a target of SUMO1 Q90A, but could be modified *in vitro* also with SUMO3.

The results for *in vivo* targets AML5 and LA are not clear. In the samples positive for modification by SUMO1 and SUMO1 Q90A the amount of unmodified substrate is slightly less compared to the negative controls. This might be a hint

for SUMOylation that leads to uptake of the substrate into higher molecular weight bands that are too weak to be detected by the used antibody.

It should also be mentioned that candidates containing at least one high probability SUMOylation consensus motif with a score above 0.90 (http://www.abgent.com/tools/sumoplot_login) could be SUMOylated with SUMO1, while none of the unmodified proteins contains such a high scoring consensus sequence.

Nevertheless, proteins that had no SUMOylation consensus motif at all like the TAF7, a TATA binding protein associated factor, or the transcription factor SVP, which only possesses consensus sequences with a low probability, were modified *in vitro*.

Both proteins contain so-called SUMO interacting motifs (SIMs), which consist of a four residues long hydrophobic stretch that can interact with the SUMO moiety. In contrast to this, the AML5 protein that has neither a high probability consensus site nor a SIM is not clearly positive for *in vitro* SUMOylation. The protein Hap2a, that was not SUMOylated at all during *in vitro* experiments, is lacking any SIM or consensus sequence as well.

2.1.2.1 **Determination of SUMOylation sites**

Due to the fact that attempts to identify SUMOylation sites of RRM1, SVP, NAF and an N-terminal fragment of CONSTANS (CO) via mass spectrometric analysis by the service unit of the MPIZ were not successful, we tried to identify the SUMOylated residues of the TAF7 protein by generating different mutant proteins, in which candidate lysine residues are exchanged. Previous MALDI-TOF analysis allowed the exclusion of a number of residues as SUMOylation sites, so that we could focus on seven lysine residues to be mutagenised.

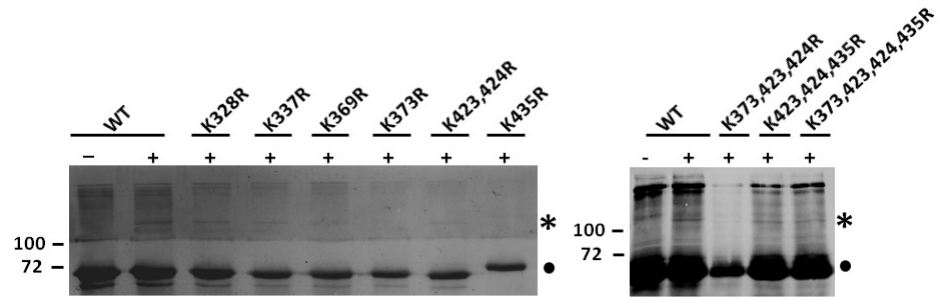


Figure 2.5: SUMOylation of mutated TAF7 proteins

To determine the residue within the TAF7 protein, to which the SUMO moiety is attached, several mutated variants with amino acid changes of lysine to arginine were generated. The proteins were analyzed for their *in vitro* SUMOylation with SUMO1 using human recombinant E1 and E2 (BostonBiochem) and a functional fragment of the *Arabidopsis* SUMO ligase SIZ1.

As shown above, TAF7 proteins that are mutated in the lysine residues K328, K337 or K369 do not differ significantly in their SUMOylation compared to TAF7 wild type protein. TAF7 variants TAF7(K373R), TAF7(K423,424R) and TAF7(K435) that are mutated in the lysine residues closer to the amino terminus, show a weaker modification by SUMO1, but are SUMOylated nevertheless.

To investigate the role of these residues further, triple or quadruple mutants were created. The proteins TAF7 (K373,423,424R) and the TAF7(K373,423,424,435R) were SUMOylated as efficiently as the wild type protein. For TAF7(K423,424,435R) no SUMOylation was detected in the shown experiment, but in this case less substrate was used.

2.2 SUMO1 Q90A, a mutant variant differing in a conserved residue

Usually, SUMO isoforms have a conserved glutamine residue at position -4 from the carboxyl terminus. The *Arabidopsis* SUMO3 and SUMO5 differ from this consensus and have a methionine and a leucine at this position, respectively, as demonstrated below (Figure 2.6).

```
SUMO1          LDMEDGDEIDAMLHQTGG/SGGGATA
SUMO1 Q90A     LDMEDGDEIDAMLHATGG/SGGGATA
SUMO2          LEMEDGDEIDAMLHQTGG/GAKNGLKLFCF
SUMO3          LDMEDGDVIDACRAMSGG/LRANQRQWSYMLFDHNGL
SUMO5          LHMEEEDEICMVMELGGG/GPYTP
```

Figure 2.6: *Arabidopsis* SUMO isoforms differ in a conserved residue.

Above, the carboxyl termini of *Arabidopsis* SUMO isoforms, which are significantly expressed, as well as the mutant variant SUMO1 Q90A are shown.
Amino acids that are generally conserved in the SUMO core, are highlighted in yellow. Differences from the conserved glutamine residue at position -4 from the carboxyl terminus are depicted in red.

Although SUMO3 and SUMO5 lack this conserved residue, these SUMO variants are significantly expressed and seem to be conjugated to substrates *in vivo* (Budhiraja, 2005). To further investigate the role of the glutamine residue at this position, several mutant plants, in which this residue was mutated in the background of SUMO1, were created in our laboratory and investigated by Dr. Ruchika Budhiraja and Dr. Andreas Bachmair. They demonstrated similar effects of the exchange of the conserved glutamine to a hydrophobic residue (leucine) or to the small amino acid alanine. The mutation led to poor growth and early senescence, finally resulting in premature death of the plant, if expressed under a constitutive promoter. Under an inducible promoter, this phenotype was less severe and an accumulation of SUMO conjugates for SUMO1 Q90A was observed compared to plants overexpressing wild type SUMO1.

Therefore, I further investigated the features of SUMO1 Q90A.

2.2.1 SUMOylation by SUMO1 Q90A

First of all, the ability of SUMO1 Q90A to modify different substrates *in vitro* was analyzed. As shown in Figure 2.4 and Table 2.1, all targets, which could be SUMOylated by SUMO1, were also modified by SUMO1 Q90A to a similar extent and in an identical pattern. Both SUMO1 varieties did also not differ during *in vitro* SUMO chain formation as presented in Figure 2.2. Dr. Ruchika Budhiraja already presented the formation of SUMO1 Q90A in plants (Budhiraja, 2005) and I analyzed the incorporation of this mutant variety after heat shock treatment of seedlings demonstrated in Figure 2.7.

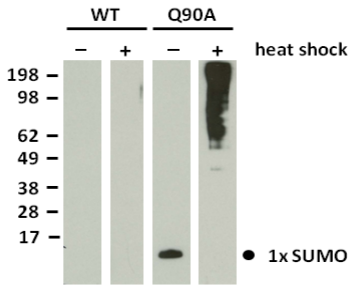


Figure 2.7: Incorporation of SUMO1 Q90A after heat shock

Col0 WT or seedlings carrying the construct pHi-SUMO1 Q90A, from which SUMO1 Q90A with a hexa-Histidine tag and three HA epitopes is constitutively expressed under control of the 35S promoter, were analyzed prior and after heat shock treatment of 1 h at 37°C. Plant extracts were analyzed by Western blot using anti-HA primary antibody and secondary anti-rat antibody coupled to horse radish peroxidase. Free SUMO is indicated by a dot.

As shown above in Figure 2.7, seedlings that were constitutively expressing the HA-tagged SUMO1 Q90A incorporated this mutant SUMO1 variant into higher molecular weight bands after heat shock treatment. Prior to the stress treatment

only the mono-SUMO moiety but no conjugates were observed. Analysis of Col0 WT control plants showed no signals with the antibodies used.

2.2.2 De-SUMOylation of SUMO1 Q90A conjugates

Similar to the behavior during SUMOylation, the features of SUMO1 Q90A regarding de-SUMOylation were analyzed. Therefore the activity of the SUMO protease ESD4 towards a SUMO1 and SUMO1 Q90A conjugate was monitored as shown in Figure 2.8.

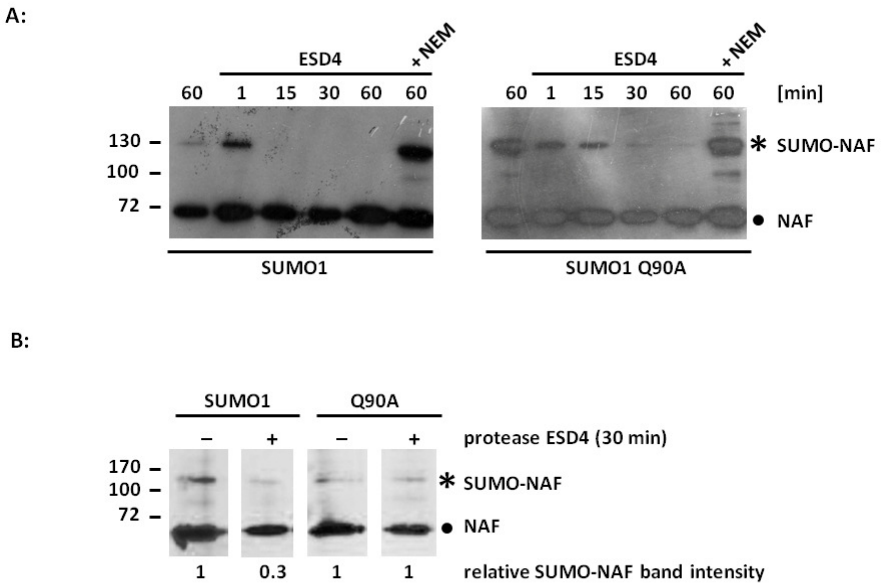


Figure 2.8: Cleavage of SUMO1 and SUMO1 Q90A conjugates by ESD4

The activity of a functional fragment of the SUMO protease ESD4 torwards SUMO1 and SUMO1 Q90A modified NAF was analyzed. Therefore a SUMOylation assay was carried out and modified and unmodified NAF were co-purified and incubated with ESD4. Aliquots of the protease reaction were separated by SDS-PAGE and monitored by Western blot with antibodies against the Flag epitope.
In the upper panel, a time course over one hour is shown, for which secondary HRP coupled antibody was used.
In the lower panel, less protease was utilized and the usage of secondary IR-dye coupled antibody allowed quantification of the experiment.
In both cases reactions were stopped by addition of 2 mM N-ethyl maleimide.

As shown above, the model substrate NAF, carrying a Flag tag, was either SUMOylated with SUMO1 or SUMO1 Q90A. Afterwards modified and

unmodified substrate were co-purified via the Flag epitope and incubated with a functional fragment of the SUMO protease Early in Short Days 4 (ESD4). In the upper panel, the activity of ESD4 against SUMO1-NAF and SUMO1 Q90A-NAF is shown. Over the analyzed time course of one hour, ESD4 is able to cleave all of the SUMO1 modified protein, but this reaction can be prevented by addition of the cysteine protease inhibitor N-ethylmaleimide (NEM). Under the same conditions, SUMO1 Q90A cleavage is far slower, but takes place nevertheless.

In the lower panel, a similar experiment is shown, but the use of IR-dye coupled secondary antibody allowed quantitative analysis of conjugate cleavage and less ESD4 was used. In this case, SUMO1 Q90A-NAF was not degraded within 30 min, while during this time approximately 70% of the applied SUMO1-NAF conjugate was cleaved.

2.3 PIAS-like SUMO ligases in *Arabidopsis*

In this work, not only *in vitro* experiments were conducted, but different plants mutated in SUMO ligases were analyzed as well.

The *Arabidopsis* genome encodes three potential SUMO ligases of the SIZ/PIAS family, SIZ1 (At5g60410), PIAS-Like1 (PIL1, At1g08910) and PIAS-Like2 (PIL2, At5g41580) (Novatchkova *et al*., 2004).

These types of proteins are known SUMO ligases in yeast and animals and are characterized by their SP-RING domain (Johnson & Gupta, 2001). They show similarity to Ubiquitin ligases of the RING type and can interact with the SCE-SUMO conjugate via their SP-RING domain.

While the importance of SIZ1 for various developmental traits and responses to biotic and abiotic factors of *Arabidopsis* is clear, the role of PIAS-Like1 and PIAS-

Like2 has not yet been analyzed. Therefore we investigated these two potential SUMO ligases further.

2.3.1 PIAS-Like1 and PIAS-Like2 differ from SIZ1 in their domain structure

SIZ1, PIL1 and PIL2 all belong to the SIZ/PIAS protein family and show some sequence homology as shown in the alignment in Figure 2.9.

Figure 2.9: Alignment of *Arabidopsis* SUMO ligases of the SIZ/PIAS family

The protein sequence of the three members of the *Arabidopsis* SIZ/PIAS SUMO ligases is shown. The SAP and PHD domains of SIZ1 (At5g60410) are indicated by a black and a grey line, respectively. The zf-MIZ finger (SP-RING) common to all three proteins is underlined in red. Domains were detected with the PFAM webservice (http://pfam.sanger.ac.uk).
In panel A, identical residues are highlighted in yellow while conservative amino acid exchanges are marked in blue. To show the similarity between PIL1 (At1g08910) and PIL2 (At5g41580), residues identical or conserved only in these two proteins are also colored (yellow).
Below in panel B, SUMOylation consensus motifs with a score ≥ 0.90 are shown in red letters, those with a probability of ≥ 0.65 in blue. Potential SUMO interaction motifs (SIMs) with the sequence V/I/L x V/I/L V/I/L or V/I/L V/I/L x V/I/L are indicated by green letters. SUMO consensus motifs were determined with the SUMOplot tool (http://www.abgent.com/tools/sumoplot).

A: Conserved residues of *Arabidopsis* SUMO ligases of the PIAS family

B: SUMOylation consensus sites and potential SUMO interaction motifs

```
SIZ1  MDLEANCKEKLSYFRIKELKDVLTQLGLSKQGKKQELVDRILTLLSDEQAARLLSKKNTVAKEAV
PIL1                  MVIPATSRFGFRAEFNTKEFQASCISLANFTENFTPGFGECSEID---
PIL2                MSTAAAARPVAGTGLREKTAASLVNSFRLASVTQRLRYHIQDGAKVDPKE

SIZ1  AKLVDDTYRKMQVSGASDLASKGQVSSDTSNLKVKGEPEDPFQPEIKVRCVCGNSLETDSMIQCE
PIL1  -------------AAIGRNEVPGNIQELALILNN---------------------------VCR
PIL2  FQICCISFAKGIDFAIANNDIPKKVEEFPWLLKQ-----------------------------LCR

SIZ1  DPRCHVWQHVGCVILPDKPMDGNPPLPESFYCEICRLTRADPFWVTVAHPLSPVRLTATTIPNDG
PIL1  RKCDDYQTRAVVMALMISVKSACQLGWFPERETQELLAIIDLMWNGFSCPEN----VTSCVNSPV
PIL2  -HGTDVYTKTALMVLMISVKHACHLGWFSDSESQELIALADEIRTCFGSSGS----TSPGIKSPG

SIZ1  ASTMQSVERTFQITRADKDLLAKPEYDVQAWCMLLNDKVLFRMQWPQYADLQVNGVPVRAINRPG
PIL1  TLISQVIERFYPCVKLGHILVS---FEAKPESKMMMKDFHISKKMPHSPKQKVGLFVVRTEDISR
PIL2  STFSQIMERFYPFVKLGHVLVS---FEVKAGYTMLAHDFYISKNMPHSLQEKIRLFVAQTDNIDT

SIZ1  GQLLGVNGRDDGPIITSCIRDGVNRISLSGGDVRIFCFGVRLVKRRTLQQVLNLIPEEGKGETFE
PIL1  SNCIVHPQGVSFLLNGKGIDKRVNISMESGPQLPTNVTALLNLGANLLQAIGCFGGSYLIAIAFM
PIL2  SACISNPPEVSFLLNGKGVEKRVNIAMDTGPQLPTNVTAQLKYGTNLLQVMGNFKGNYIIIIAFT

SIZ1  DALAR-VRRCIGGGGGDDNADSDSDIEVVADFFGVNLRCPMSGSRIKVAGRFLPCVHMGCFDLDV
PIL1  DVIPLPNKPLLKDYVHPEVVGSNSDCDIIEGPSRISLSCPISRTRIKLPVKGHVCKHLQCFDFWN
PIL2  GLVVPPEKPVLKDYLQSGVIEASPDSDIIEGPSRVSLSCPISRKRIKLPVKGQLCKHLQCFDFSN

SIZ1  FVELNQRSRKWQCPICLKNYSVEHVIVDPYFNRITSKMKHCDEEVTEIEVKPDGSWRVKFKRESE
PIL1  YVNMNTR-----------------RHHGAARIILEEVGRN---AADVVISADGTWMVETENDED
PIL2  YVHINMRNPTWRCPHCNQPVCYPDIRLDQNMAKILKDVEHN---AADVIIDAGGTWKVTKNTGET

SIZ1  RRELGELSQWHAPDGSLCPSAVDIKRKMEMLPVKQEGYSDGPAPLKLGIRKNRNGIWEVSKPNTN
PIL1  VELVPETTHDHGDPNSFINLGPTVKNPARD---ENEMETSTQVEEHNPCLSEIQGPSN----DTH
PIL2  PEPVREIIHDLEDPMSLLNSGPVVFDLTGDDDAELEVFGDNKVEDRKPCMSDAQGQSNNNNTNKH

SIZ1  GLSSSNRQEKVGYQEKNIIPMSSSATGSGRDGDDASVNQDAIGTFDFVANGMELDSISMNVDSGY
PIL1  RPASDYTMLNQS----------HTSTNTLPQLPRTLNAFDGQQFVNLPQVINTRDSPASQALPM
PIL2  PSNDDYSSIFDISDVIALDPEILSALGNTAPQPHQASNTGTGQQYSNLSQIPMSIDP---MPVPV

SIZ1  NFPDRNQSGEGGNNEVIVLSDSDDENDLVITPGPAYSGCQTDGGLTFPLNPPGIINSYNEDPHSI
PIL1  TFSPTPSPQDILATNAANFGTSMPAAQSSQFQGSHVTSLGNCEGRTSDLMAR-WNHIYGRVQTQF
PIL2  PFSQTPSPRDRPATTSTVFTIPNPSPQYSQVHASPVTPTGTYLGRT--TSPR-WNQTY-----QS

SIZ1  AGGSSGLGLFNDDDEFDTPLWSFPSETPEAPGFQLFRSDADVSGGLVGLHHHSPLNCSPEINGGY
PIL1  PPAPLSHHHYSMQNQSPSPAQQRPVPSYIAHPQTFHVNYGENADQRWMPSSIAHPQTLPVNYGGN
PIL2  QAPPMTTPYTSRKVSVPVTSQS-----------------------------------PANVSSF

SIZ1  TMAPETSMASVPVVPG----------------STGRSEANDGLVDNPLAFGRDDPSLQIFLPTK
PIL1  TNQRPIPSSIAHPQTLPVNYRGNTDHRSTPYSITHLQTLLNYGGNADQRPMPSSITNLQTLPATY
PIL2  VQSQHVPRVLSQPN-------------------------NYG--------------VRGLTSSH

SIZ1  PDASAQSGFKNQADMS-------------------------------------------------NGLRS
PIL1  GGYAHQRPMSSSITHPRTSPVNYGGTPDQRPMPSSITHPQTLPVSYGGTTDQILNPGGAMGQFSS
PIL2  ASTSRQHPSGPTVQS-----------------------------------------------VSRLSD

SIZ1  EDWISLRLGDSASGNHGDPATTNGINSSHQMSTREGSMDTTTETASLLLGMNDSRQDKAKKQRSD
PIL1  REFMNLTPANTENWRPQSRMRGSVAPG---TGYDHMIIHPTRPVHPQAQTPPAPLSTSYDGADEI
PIL2  LVDVDLTVPDTSNWRP--RMRGSLVPGSHSTALDHMIIRPSQ--QSQTSTRLNSSQPVQTPSVQT

SIZ1  NPFSFPRQKRSVRPRMYLSIDSDSETMNRIIRQDTGV------
PIL1  QAFIGHPSYPVSNNETQAGTSSLPVAEGLGYSGSFWSMPPETW
PIL2  SQAQSPFTTAAYRTETVLGNRNHPVPAPPGIVRPTGPTS----
```

As demonstrated above in Figure 2.9, SIZ1 has in contrast to PIL1 and PIL2 two additional domains: a SAP domain and a PHD finger. It is also striking that SIZ1 possesses different high probability SUMOylation consensus motifs that are absent in the two other proteins.

Nevertheless, all three proteins contain SP-RING domains. This domain type is also called zf-MIZ and confers SUMO ligase activity and shows similarity to the RING domain found in Ubiquitin ligases (Cheng *et al*., 2007; Wu *et al*., 1997; Zhao & Blobel, 2005). The occurrence of this domain indicates a SUMO ligase function. In contrast to the other two proteins, the potential zf-MIZ domain of PIL1 is slightly shorter and lacks two cysteines and a proline residue close to the end of the domain that are conserved in SIZ1 and PIL2. To investigate the potential SUMO ligase function of PIL1 and PIL2, the amount of SUMO conjugates in the respective mutants was analyzed.

2.3.2 SUMO conjugates of SUMO ligase mutants

The SP-RING domain of the PIAS-Like family suggests a SUMO ligase function of these proteins. To analyze this hypothesis, the amount of SUMO conjugates of different PIAS-like mutant plants was analyzed as shown in Figure 2.10, because the knockout of a SUMO ligase could lead to a decrease in SUMO conjugates.

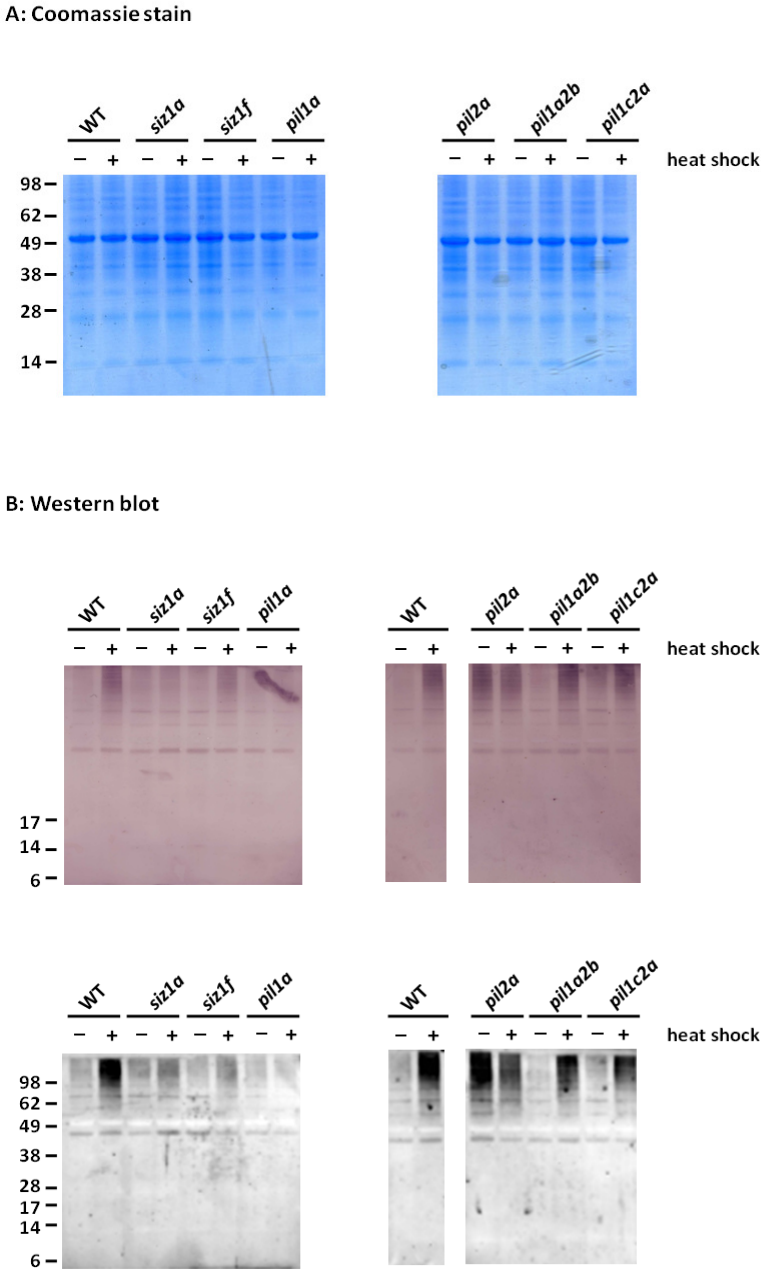


Figure 2.10: SUMO1 conjugates of different SUMO ligase candidate mutants

The total protein amount of plant extracts of different SUMO ligase mutants was detected by Coomassie stain (panel A). Afterwards, equal protein amounts were tested for SUMO conjugates as demonstrated in panel B. Therefore an aliquot of plant extract prepared under denaturing conditions was separated via SDS-PAGE. SUMO1 conjugates were detected with anti-SUMO1 antibody (ABCAM) and secondary anti rabbit antibody coupled to alkaline phosphatase (upper panel) or horse radish peroxidase (lower panel). The experiment with horse radish peroxidase coupled antibody was performed together with Dr. Kishore Panigrahi, MPIZ, and signals were detected with the LumiImager system. Untreated plants were compared to those that were heat shocked for an hour at 37°C.

In Figure 2.10, the detection of SUMO1 conjugates of different SUMO ligase mutants is shown. Untreated plants and those that underwent a heat shock treatment for an hour at 37°C were compared. In wild type plants under normal conditions few higher molecular weight bands were detected, indicating a low level of SUMO conjugates. The amount of these conjugates increased strongly after heat shock treatment. *Siz1* mutant plants show fewer conjugates and do not respond to heat shock as strongly as wild type. The weaker allele *siz1f* shows a higher amount of SUMO conjugates after stress treatment compared to the *siz1a* mutant that has a stronger phenotype.

In the *pil1* mutant the overall amount of SUMO conjugates is slightly decreased compared to the untreated wild type and the level of conjugates does not differ significantly with and without heat shock treatment. In contrast to this, the *pil2* mutant shows an accumulation of SUMO conjugates under normal conditions that equals the amount after heat shock treatment both in *pil2* and the wild type control plant. The *pil1pil2* double mutants have slightly reduced SUMO conjugates under normal conditions and show an increase of SUMO modified proteins after stress treatment that is weaker than those observed in wild type but significantly stronger than in the *siz1* mutants.

2.3.3 Phenotypic analysis of *pil* mutant plants

To investigate the role of PIL proteins in plant development, the growth and flowering phenotype of different *pil* single and double mutants was monitored. Figure 2.11 shows plants grown under long day and short day conditions.

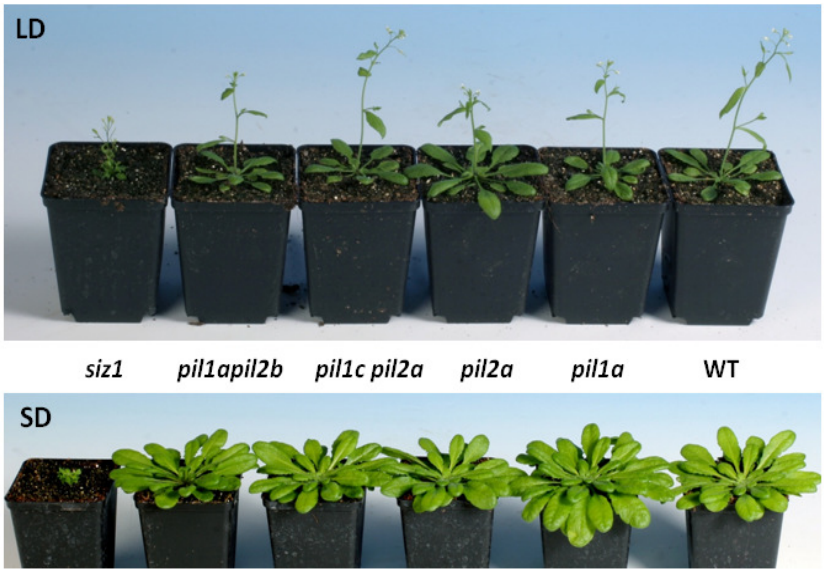


Figure 2.11: SUMO ligase mutant plants grown under different light conditions

Above different SUMO ligase mutants that were grown either under long day conditions (16 hours light) or short day conditions (eight hours light), are shown.

As demonstrated in Figure 2.11, *siz1* mutant plants are dwarfish and bushy as described before. In contrast to this, the *pil1* and *pil2* single mutants as well as the two analyzed lines of *pil1pil2* double mutants cannot be distinguished from the wildtype control under both long day and short day conditions.

Additionally, the flowering time of the different ligase mutants was monitored under different light conditions, because it was already known that s*iz1* mutants flower early (Jin *et al*., 2008). The results of these experiments are listed below.

Table 2.2: **Flowering time analysis of different SUMO ligase mutants**

		rosette leaves			cauline leaves		
light	**genotype**	**mean**	**st dev**	**significance**	**mean**	**st dev**	**significance**
LD	Col 0 WT	11,29	1,45		1,62	0,81	
	siz1a	11,11	1,69	no	1,67	1,00	no
	siz1c	9,33	1,33	yes	1,72	0,58	no
	siz1f	nt	nt	nt	nt	nt	nt
	pil1apil2b	10,95	1,64	no	2,20	0,52	no
	pil1cpil2a	11,00	1,58	no	6,04	0,50	no
LD	Col 0 WT	28,57	10,55		3,86	1,23	
	siz1a	9,77	3,46	yes	3,29	0,77	no
	siz1c	10,00	4,62	yes	3,06	0,75	no
	siz1f	20,94	5,41	no	4,00	0,08	no
	pil1apil2b	25,77	6,92	no	4,18	0,95	no
	pil1cpil2a	25,82	7,33	no	6,14	0,09	no
ex SD	Col 0 WT	42,33	5,09		6,00	1,00	
	siz1a	28,14	8,24	yes	4,38	1,88	yes
	siz1c	30,86	7,33	yes	4,33	1,24	yes
	siz1f	nt	nt	nt	nt	nt	nt
	pil1apil2b	36,52	5,21	no	5,91	1,04	no
	pil1cpil2a	45,52	4,71	no	6,25	0,66	no
SD	Col 0 WT	63,14	23,08		6,71	2,27	
	siz1a	52,47	21,21	no	5,60	1,55	no
	siz1c	61,94	20,18	no	5,31	1,20	no
	siz1f	92,06	11,63	yes	9,44	2,31	yes
	pil1apil2b	86,94	11,09	yes	8,59	1,00	no
	pil1cpil2a	92,18	12,44	yes	6,36	0,95	yes

The results of a one way comparison conducted with SigmaPlot 10 and SigmaStat 3 are shown. An overall significance level of 0.01 was chosen.

For an easier overview, the results of these flowering time experiments are shown graphically in Figure 2.12.

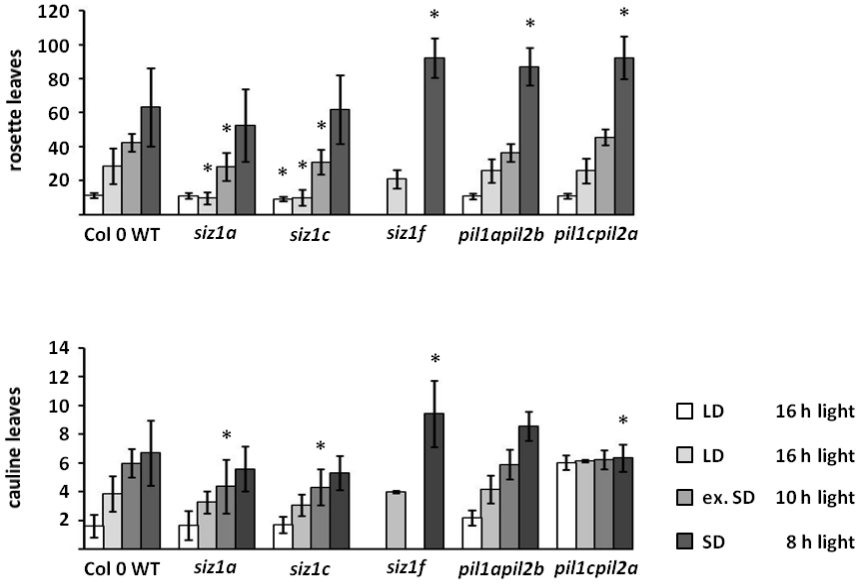


Figure 2.12: Flowering time of different SUMO ligase mutants

The flowering time of different *siz1* mutant lines and the *pil1pil2* double mutants was analyzed under various light conditions as indicated. Rosette and cauline leaves were counted at the time of opening of the first flower bud. A one way comparison of the data was carried out with an overall significance level of 0.01. Bars represent mean numbers of leaves for different light regimes, with standard deviation indicated as arrow bars. Values that differed significantly from wild type are marked by an asterisk above the respective bar.

The flowering time of the different SUMO ligase mutants was analyzed under various light conditions. Two experiments were performed under long day conditions with 16 hours of light per day and single data sets were available for extended short day (10 hours of light) and short day conditions with eight hours light per day.

Under long day condition, an early flowering of *siz1c* line in both experiments and of *siz1a* in one experiment was observed. In contrast to these two lines, *siz1f* did not flower significantly earlier compared to the Columbia wild type in long day. At ten hours of light per day (extended short day), the tested *siz1a* and *siz1c* mutants also flowered earlier and had on average two cauline leaves less compared to wild type. These differences were not observed under eight hours

light per day (short day) but in this case, the *siz1f* plants flowered later as the wild type by nearly thirty rosette leaves. Additionally, *siz1f* produced more cauline leaves during this experiment.(Jin *et al*., 2008b)

The *pil1pil2* double mutants differed only significantly in short day from the Columbia wild type. On average, the *pil1apil2b* and *pil1cpil2a* possessed from more than twenty up to nearly thirty rosette leaves more under this condition and the *pil1cpil2a* line produced excess cauline leaves as well.

2.4 The *Arabidopsis* SUMO protease Early in Short Days-Like 1 (EL1)

Not only SUMO ligases play an important role during SUMOylation. Another class of proteins regulating SUMOylation are the SUMO proteases, because they control the abundance of mature SUMO moieties and can de-SUMOylate substrates.

In *Arabidopsis* several genes encoding potential SUMO proteases are found (Colby *et al*., 2006; Novatchkova *et al*., 2004). Three of them show similarity to the yeast SUMO protease Ulp1. One of these, named Early in Short Days 4 (ESD4, At4g15880) has already been described (Murtas *et al*., 2003; Reeves *et al*., 2002). It is involved in flowering time control and is located mainly at the inner side of the nuclear envelope (Murtas *et al*., 2003; Reeves *et al*., 2002).

The other two members with similarity to the yeast SUMO protease Ulp1, the Ulp1b (At4g00690) and Ulp1a (At3g06910) are less well described. However, a close homology of Ulp1a to ESD4 was shown, so it is also named Early in Short Days 4 –Like 1 (EL1) and Dr. Yong Fu-Fu in the laboratory of Prof. George Coupland demonstrated *in vitro* SUMO protease activity for this protein (Yong Fu-Fu, unpublished data).

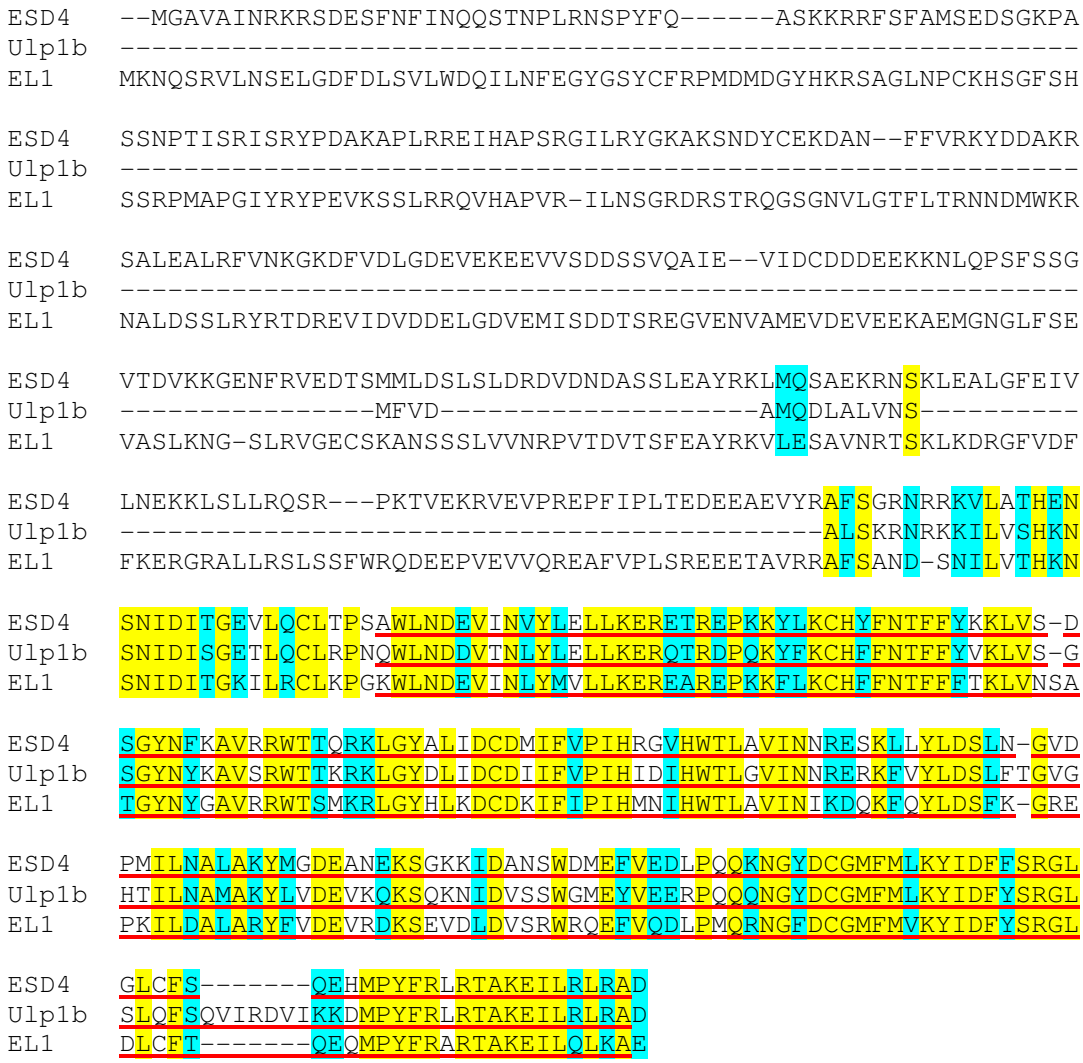
In this work, the role of EL1 (Ulp1a) was further investigated.

2.4.1 Domain structure of El1

EL1 and Ulp1b are the closest homologs of ESD4 in *Arabidopsis*.

In Figure 2.13, an alignment of all three Ulp1-related open reading frames of *Arabidopsis* is shown.

A: Residues, conserved in the Ulp1a type SUMO proteases of *Arabidopsis*



B: SUMOylation consensus sites and potential SUMO interaction motifs

```
ESD4    --MGAVAINRKRSDESFNFINQQSTNPLRNSPYFQ------ASKKRRFSFAMSEDSGKPA
Ulp1b   ------------------------------------------------------------
EL1     MKNQSRVLNSELGDFDLSVLWDQILNFEGYGSYCFRPMDMDGYHKRSAGLNPCKHSGFSH

ESD4    SSNPTISRISRYPDAKAPLRREIHAPSRGILRYGKAKSNDYCEKDAN--FFVRKYDDAKR
Ulp1b   ------------------------------------------------------------
EL1     SSRPMAPGIYRYPEVKSSLRRQVHAPVR-ILNSGRDRSTRQGSGNVLGTFLTRNNDMWKR

ESD4    SALEALRFVNKGKDFVDLGDEVEKEEVVSDDSSVQAIE--VIDCDDDEEKKNLQPSFSSG
Ulp1b   ------------------------------------------------------------
EL1     NALDSSLRYRTDREVIDVDDELGDVEMISDDTSREGVENVAMEVDEVEEKAEMGNGLFSE

ESD4    VTDVKKGENFRVEDTSMMLDSLSLDRDVDNDASSLEAYRKLMQSAEKRNSKLEALGFEIV
Ulp1b   ----------------MFVD--------------------AMQDLALVNS----------
EL1     VASLKNG-SLRVGECSKANSSSLVVNRPVTDVTSFEAYRKVLESAVNRTSKLKDRGFVDF

ESD4    LNEKKLSLLRQSR---PKTVEKRVEVPREPFIPLTEDEEAEVYRAFSGRNRRKVLATHEN
Ulp1b   ---------------------------------------------ALSKRNRKKILVSHKN
EL1     FKERGRALLRSLSSFWRQDEEPVEVVQREAFVPLSREEETAVRRAFSAND-SNILVTHKN

ESD4    SNIDITGEVLQCLTPSAWLNDEVINVYLELLKERETREPKKYLKCHYFNTFFYKKLVS-D
Ulp1b   SNIDISGETLQCLRPNQWLNDDVTNLYLELLKERQTRDPQKYFKCHFFNTFFYVKLVS-G
EL1     SNIDITGKILRCLKPGKWLNDEVINLYMVLLKEREAREPKKFLKCHFFNTFFFTKLVNSA

ESD4    SGYNFKAVRRWTTQRKLGYALIDCDMIFVPIHRGVHWTLAVINNRESKLLYLDSLN-GVD
Ulp1b   SGYNYKAVSRWTTKRKLGYDLIDCDIIFVPIHIDIHWTLGVINNRERKFVYLDSLFTGVG
EL1     TGYNYGAVRRWTSMKRLGYHLKDCDKIFIPIHMNIHWTLAVINIKDQKFQYLDSFK-GRE

ESD4    PMILNALAKYMGDEANEKSGKKIDANSWDMEFVEDLPQQKNGYDCGMFMLKYIDFFSRGL
Ulp1b   HTILNAMAKYLVDEVKQKSQKNIDVSSWGMEYVEERPQQQNGYDCGMFMLKYIDFYSRGL
EL1     PKILDALARYFVDEVRDKSEVDLDVSRWRQEFVQDLPMQRNGFDCGMFMVKYIDFYSRGL

ESD4    GLCFS-------QEHMPYFRLRTAKEILRLRAD
Ulp1b   SLQFSQVIRDVIKKDMPYFRLRTAKEILRLRAD
EL1     DLCFT-------QEQMPYFRARTAKEILQLKAE
```

Figure 2.13: Alignment of *Arabidopsis* SUMO proteases of the Ulp1 type

An alignment of the protein sequences of the three members of the *Arabidopsis* SUMO proteases of the Ulp1 type is shown. Identical residues are highlighted in yellow while conservative amino acid changes are marked in blue in panel A.
In panel B, SUMOylation consensus motifs with a score ≥ 0.90 are shown with red letters, those with a probability of ≥ 0.65 in blue. Potential SUMO interaction motifs (SIMs) with the sequence V/I/L x V/I/L V/I/L or V/I/L V/I/L x V/I/L are indicated by green letters. SUMO consensus motifs were determined with the SUMOplot tool (http://www.abgent.com/tools/sumoplot).
The conserved Peptidase_C48 domain in all three proteins is highlighted in red in both panels and was detected with the PFAM webservice (http://pfam.sanger.ac.uk).

As shown in the alignment above, all three proteins contain a so called Peptidase_C48 domain, which is typical for cysteine proteases involved in cleavage of SUMO precursors or SUMOylated conjugates (Mossessova & Lima, 2000). Although the catalytic domains of ESD4 and Ulp1b show a high similarity, the Ulp1b protein is far smaller and lacks the amino terminal extension that is found in both ESD4 and EL1. Therefore ESD4 and EL1 have overall a higher similarity to each other than to Ulp1b and subsequent experiments focused on EL1 rather than on Ulp1b. Both ESD4 and El1 have a conserved SUMOylation consensus motif on the amino terminal side of the peptidase domain (TD**VKKG**EN, VAS**LKNG**SL), and ESD4 has an additional high probability SUMOylation site (PD**AKAP**LR) closer to the amino terminus that is not found in the other two proteases. EL1 differs from the other two proteins in its SUMO interaction motifs (SIMs). While in Ulp1b and ESD4 only a single potential SIM is found at the amino terminal side of the catalytic domain, EL1 has four potential interaction motifs in this region.

2.4.2 Localization of EL1

After attempts to express EL1 as a GFP-fusion protein in *Arabidopsis* failed, EL1-GFP constructs were transiently expressed in *Nicotiana benthamiana* leaves and their localization was detected by confocal microscopy with the help of the CeMic group at the MPIZ. The results of these experiments are shown in Figure 2.14.

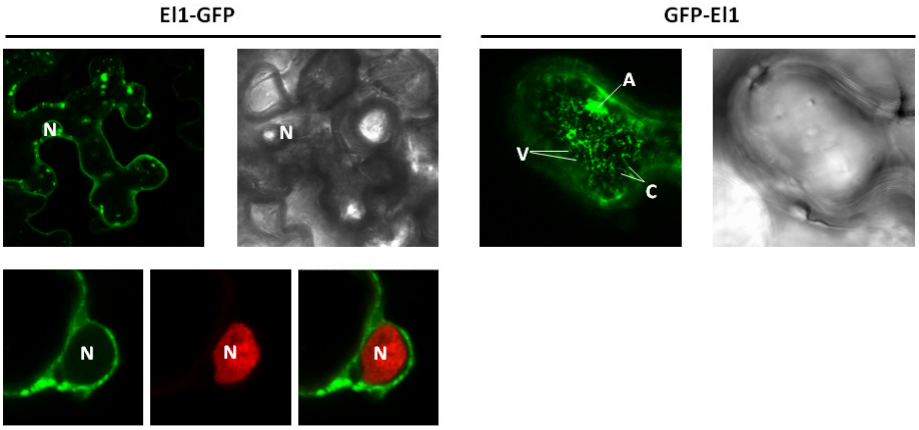


Figure 2.14: Localization of El1

Carboxyl and amino terminal fusion of El1 to GFP were transiently expressed in *Nicotiana benthamiana* leaves and detected by confocal microscopy. In the lower panel, the nucleus was stainend with propidium iodide. The experiments were conducted by Dr. Elmon Schmelzer and Rainer Franzen at the CeMic facility of the MPIZ.

N: Nucleus A: Aggregates
V: Vesicels C: Cytoplasmic strands

As demonstrated above, the localization of both EL1-GFP and GFP-EL1 was analyzed in *Nicotiana benthamiana* leaves. Both fusion proteins were almost completely absent from the nucleus, but found in vesicular structures that might be connected to cytoplasmic strands as observed for the carboxy terminal fusion protein. The aggregates observed in case of GFP-EL1 might be due to strong protein expression. The propidium iodide stain in the lower panel shows clearly that the EL1-GFP is not nuclear or at the nuclear envelope, but partially surrounds the nucleus in a structure that may be part of the ER.

2.4.3 Phenotypic analysis of *el1* mutant plants

Different *el1* mutant lines were analyzed for their growth and flowering phenotype, because it is known that mutants of the SUMO protease ESD4 flower extremely early under short days and are dwarfish and bushy.

Figure 2.15 shows various *el1* mutant lines grown under SD.

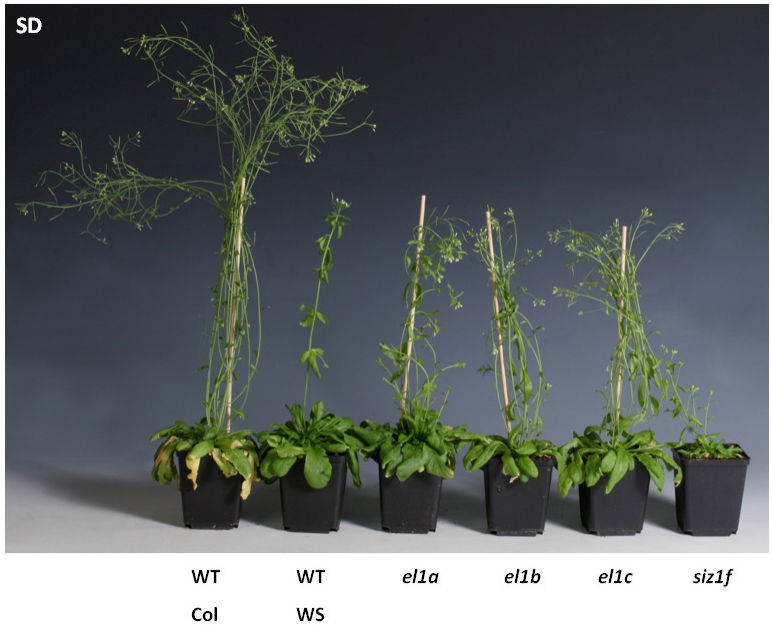


Figure 2.15: Different *el1* mutants grown under short day conditions

Comparison of the growth habit of different *el1* mutants, grown under short day conditions (eight hours light per day). As controls, Columbia and Wassilewskija wild type plants and a *siz1* mutant plant are shown as well.

In short days with eight hours light per day, a condition that leads to an extremely bushy and dwarfish phenotype of *esd4* mutants, the different tested *el1* lines do not differ strongly from wild type. All three lines reach a similar height as the Wassilewskija wild type control, and show a number of side shoots in between those of Columbia and Wassilewskija wild type. The *el1* mutants have slightly serrated leaves, which were already observed by Dr. Yong Fu-Fu for the *el1a* mutant line in the background of Wassilewskija. In case of this line, which carries a mutation in the first exon of the el1 gene, slightly thinner stems compared to wild type were observed. In contrast to this, the other lines *el1b* and *el1c* that are both in the background of the ecotype Columbia did not differ in stem thickness from wild type. To further investigate this observation, the tissue composition of stems of the *el1a* was investigated as demonstrated in Figure 2.16 .

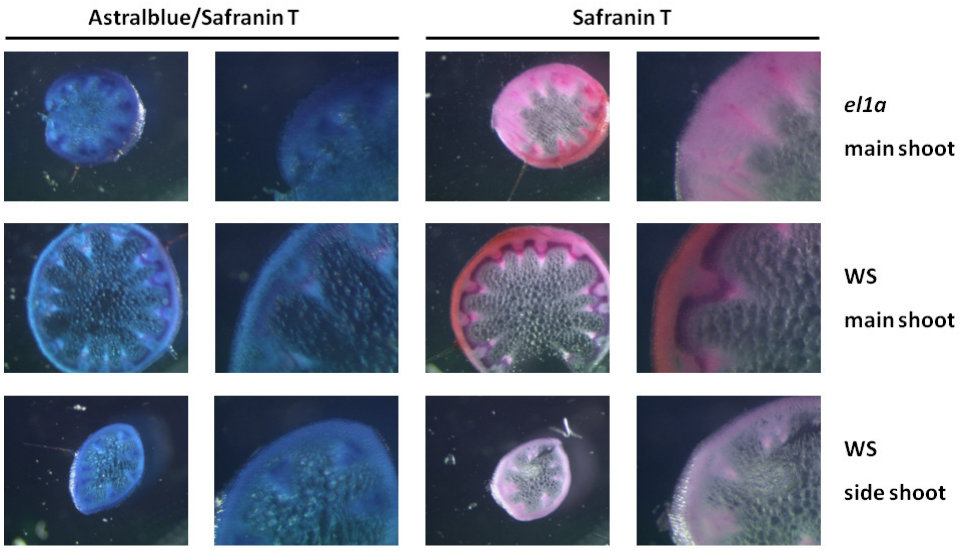


Figure 2.16: Altered tissue composition of *el1* line 1 stems

Comparison of the stem thickness and tissue composition of *el1a* and Wassilewskija (WS) wild type plants. Plants that just started to flower were utilized. The main stems were cut above the first cauline leaf and slices were stained either with Astralblue and Safranin T or only with Safranin T as indicated. All pictures are shown with the same magnification.

In Figure 2.16, the stems of *el1a* mutant plants are shown in comparison to wild type. Stems of plants of the same developmental state that just begun to flower, were cut and stained with Safranin T or Safranin T and Astralblue. The combination of the two dyes Astrablue and Safranin T (left panel) indicates that *el1*a shoots have less mark compared to Wassilewskija wild type main shoots.

To rule out that the proportional thicker vascular tissue is due to the overall size of the stem, a wild type side shoot with similar diameter was analyzed as well. In the wild type side shoot, the area of the vasculature, which is stained brightly blue by the Astralblue/ Safranin T stain, is thinner than that of the *el1* shoot with a similar thickness.

The stain with Safranin T (right panel) confirmed that the vascular tissue of the *el1a* main shoot is far thicker compared to the stem of the wild type plant, although overall the stem of *el1a* has approximately only half the diameter of the wild type one. Again, this observation cannot be explained by the total size of the shoot, because a thinner wild type side shoot possesses a significantly slimmer

outer layer compared to the *el1a*. In the main shoot of Wassilewskija, a pink ring is visible after the Safranin T staining that could be interfascicular cambium indicating secondary radial growth. A similar structure could not be observed in the analyzed *el1a* shoot.

The flowering time of the different *el1* lines was also monitored. The results of two experiments under long day conditions and a single experiment under eight hours of light are summarized in Table 2.3.

Table 2.3: **Flowering time analysis of different *el1* lines**

		rosette leaves			cauline leaves		
light	**genotype**	**mean**	**st dev**	**significance**	**mean**	**st dev**	**significance**
LD	Col 0 WT	12,18	1,60		2,64	0,51	
	WS WT	8,82	1,99		3,00	0,78	
	el1a	9,58	0,79	no	6,50	1,09	yes
	el1b	12,33	1,72	no	2,58	0,67	no
	el1c	11,09	1,92	no	2,73	0,47	no
	siz1f	10,18	1,08	no	2,82	0,75	no
LD	Col 0 WT	14,36	4,46		2,82	0,60	
	WS WT	9,60	1,43		3,30	0,48	
	el1a	10,83	0,84	no	2,67	0,49	no
	el1b	15,83	3,27	no	3,50	0,52	no
	el1c	15,33	3,23	no	3,50	0,80	no
	siz1f	13,25	3,39	no	3,25	0,62	no
SD	Col 0 WT	72,13	26,65		10,88	2,80	
	WS WT	63,00	31,71		7,64	0,81	
	el1a	48,91	24,28	no	5,82	1,60	no
	el1b	64,33	26,19	no	11,00	4,30	no
	el1c	78,80	23,34	no	11,30	3,20	no
	siz1f	44,00	16,54	no	10,67	2,18	no

The results of a one way comparison conducted with SigmaPlot 10 and SigmaStat 3 are shown. An overall significance level of 0.01 was chosen. The *el1* line1 was compared to the Wassilewskija wild type (WS WT) and the other two lines to Columbia 0, because the mutations are in the respective background.

For a better overview, these data are graphically presented in Figure 2.17.

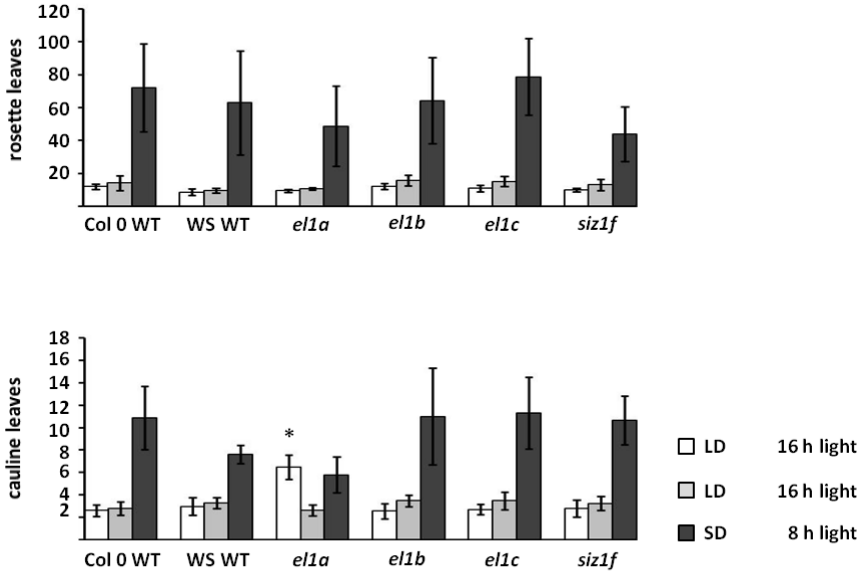


Figure 2.17: Flowering time of different *el1* lines

The flowering time of different *el1* mutant lines was monitored under long and short day conditions, with sixteen or eight hours light per day, respectively. Rosette and cauline leaves were counted at the opening time of the first flower bud. A one way comparison of the data was conducted with an overall significance level of 0.01 Above the mean number of leaves during that time point and the according standard deviation (arrow bars) are shown and values that differed significantly from the wild type under the same condition are indicated by an asterisk. As the *el1a* line is in the background of Wassilewskija and the lines *el1b* and *c* are in the background of the ecotype Columbia, the lines were compared to the respective wild type.

The analysis of the flowering time of different *el1* mutant lines is demonstrated above in Table 2.3 and summarized in Figure 2.17. In these experiments, two different ecotypes were monitored as control, because the *el1a* line is in the background of Wassilewskija while the *el1b* and *el1c* are in the Columbia background.

All three analyzed *el1* mutant lines did not differ significantly from the respective wild type in their number of rosette leaves at the opening of the first flower. Only the number of cauline leaves of *el1a* was significantly increased in comparison to Wassilewskija during one experiment under long day conditions. Nevertheless,

this was not observed in a second experiment under long day, in which the number of cauline leaves of *el1a* and the wild type control equaled.

Furthermore, *siz1f* was analyzed in parallel, but this mutant line did not differ significantly from wild type (Columbia) in any experiment.

Additionally it has to be mentioned that the standard deviation (st dev) in the experiment with short day conditions is extremely high and that this experiment was conducted with a different batch of soil.

2.5 Early in Short Days 4 (ESD4), a SUMO protease involved in flowering time control

As mentioned before, Early in Short Days 4 (ESD4, At4g15880) is a SUMO protease of the Ulp1 type, which is involved in the control of flowering time (Murtas *et al*., 2003; Reeves *et al*., 2002). Probably, ESD4 can delay flowering via control of FLC, a floral repressor, and mutants of this SUMO protease flower extremely early under short day conditions (Murtas *et al*., 2003; Reeves *et al*., 2002). This protein is mainly nuclear and enriched at the inner side of the nuclear envelope. It contains the Peptidase_C48 domain (Figure 2.13) that is typical for SUMO proteases of the Ulp1 type, and its SUMO protease activity has already been demonstrated (Murtas *et al*., 2003; Reeves *et al*., 2002).

The *esd4* mutants do not only flower early, but have a reduced apical dominance and growth. This observation resembles the phenotype of the bushy and dwarfish *siz1* mutant plants. As it is known that the growth defect of *siz1* is at least partly due to an increased level of salicylic acid in those mutant plants, it was assumed that an exaggerated amount of this phyto hormone in e*sd4* might (partly) cause the observed growth phenotype. Therefore *esd4* mutant plants were crossed with *sid2* plants that are defective in salicylic acid biosynthesis. The offspring of this cross was monitored for rescue of the *esd4* induced growth effect due to decreased salicylic acid levels by the introduced *sid2* mutation.

Figure 2.18 shows the growth phenotype of those plants.

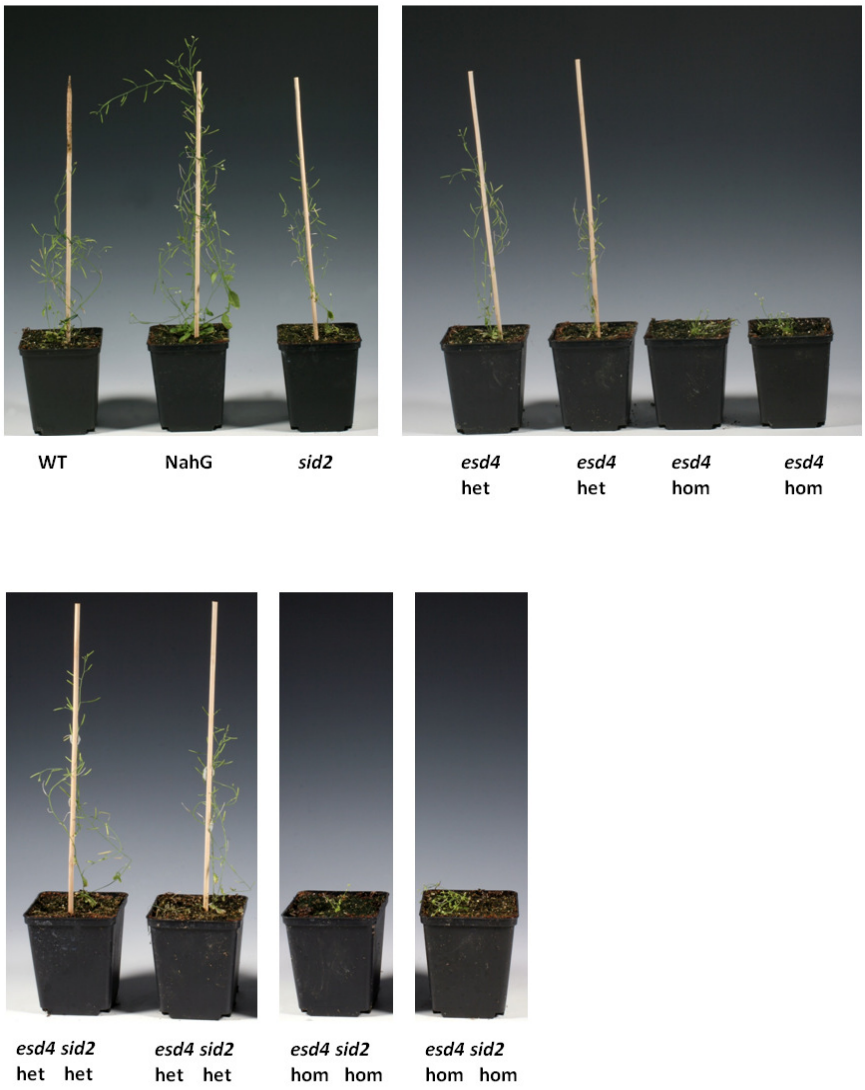


Figure 2.18: Phenotype of *esd4 sid2* plants

Wild type, *sid2* and *esd4* homozygous (hom) and heterozygous (het) mutants grown under LD conditions are shown in the upper panel. Below, different crosses of *esd4* to *sid2* are shown. The plants were grown in summer time during a rather warm period.

As already mentioned, *esd4* homozygous mutants are dwarfish and extremely bushy even under long day conditions. This extreme growth phenotype is not so pronounced in the heterozygous *esd4* plants, which are significantly bigger than the homozygous mutants and less arborescent.

A similar result was obtained for the cross of *esd4* plants with a *sid2* line. The resultant *esd4(hom)sid2(hom)* and *esd4(het)sid2(het)* offspring showed a similar growth phenotype as the *esd4* homo- or heterozygous plants, respectively.

2.6 The bacterial effector protein Factor X

It is known that some pathogens try to undermine the resistance system of their host by modulating post-translational protein modification in the host cell (Janjusevic *et al*., 2006; Orth *et al*., 2000). Some of these bacterial virulence factors possess Ubiquitin or SUMO protease activity towards host proteins (Angot *et al*., 2007; Orth *et al*., 2000). It has been demonstrated that plant pathogens produce different virulence factors, which are secreted into plant cells via a bacterial type III secretion system. Some of these effectors possess SUMO protease activity (Gurlebeck *et al*., 2006; Hotson *et al*., 2003; Roden *et al*., 2004).
In cooperation with Prof. Ulla Bonas and Robert Szczesny from the Martin-Luther-University in Halle, Germany, the bacterial effector Factor X from the pathogen *Xanthomonas campestris* was tested for *in vitro* SUMO and Ubiquitin protease activity.

2.6.1 *In vitro* protease activity of Factor X

To test the ability of Factor X to cleave Ubiquitin and SUMO conjugates, this potential protease was produced as a recombinant protein in *E. coli* and its activity against different fusion proteins was tested. The results of this experiment are shown in Figure 2.19.

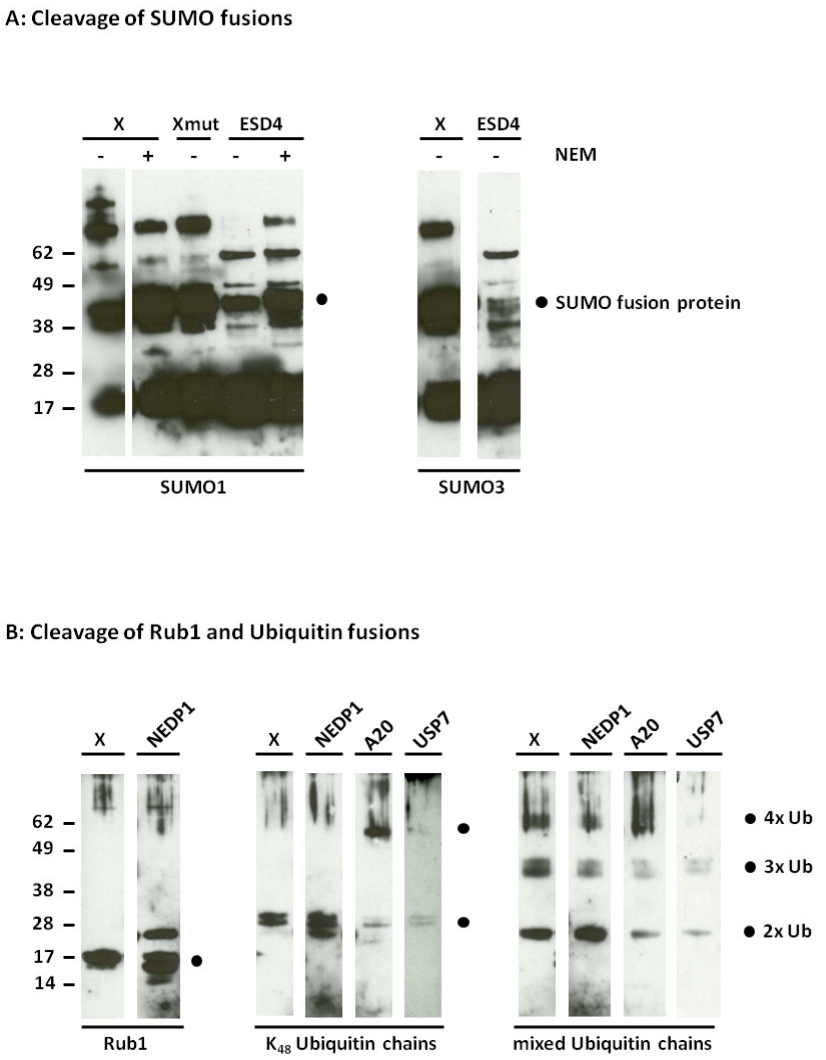


Figure 2.19: Test for protease activity of Factor X

Recombinant Factor X, a mutant variant of Factor X, in which the potential catalytic Cysteine is mutated, or a fragment of the SUMO protease ESD4 were incubated with SUMO1 or SUMO3 fused to the transcription factor FLC. The lower panel shows incubations to probe the ability of Factor X to cleave a recombinant Rub1 (Nedd8) fusion protein or commercially available Ubiquitin chains with a K48 or mixed linkages (BostonBiochem). As controls, human recombinant proteases NEDP1 (a Nedd8 protease), A20 and USP7 (two Ubiquitin proteases) were used (BostonBiochem). The samples were incubated in ATP and zinc containing buffer overnight at 30°C. Reactions were stopped by adding the cysteine protease inhibitor N-ethylmaleimide (NEM) to a final concentration of 2 mM, in negative controls (upper panel) the inhibitor was added prior to incubation. An aliquot of the reaction mixture was separated by SDS-PAGE and analyzed via Western blot with antibody against SUMO1 (ABCAM) or anti-Ubiquitin serum (A. Bachmair). Secondary antibody coupled to horse radish peroxidase was used for visualization of substrates.

As shown above, the ability of Factor X to cleave SUMO fusion proteins was tested. In case of the SUMO1 fusion protein, the sample with potentially active Factor X does not differ significantly from the control with Factor X mut, in which the proposed catalytically active cysteine residue is mutated, or from the negative control in which protease inhibitor (NEM) was added prior to incubation. In contrast to this, a functional fragment of ESD4 was able to cleave the SUMO1 fusion protein. This reaction could be inhibited by the cysteine protease inhibitor N-ethylmaleimide. Similar results were obtained for SUMO3. Again no protease activity was observed for Factor X, while the ESD4 fragment cleaved the SUMO3 fusion protein.

The activity of Factor X towards other protein modifiers was analyzed as well. Factor X showed no activity against fusion proteins with Rub1, the *Arabidopsis* orthologue to the mammalian Nedd8. Whereas commercially available, human recombinant Nedd8 protease NEDP1 cleaved this construct, indicated by the occurrence of a smaller molecular weight band representing the fusion protein without Rub1.

Different Ubiquitin chains were also tested. Factor X was not able to cleave K_{48}-linked Ubiquitin chains, which were degraded by NEDP1 and human recombinant A20 as indicated by the occurrence of a band with smaller molecular weight. In case of USP7 no smaller weight band occurred, but in general this lane stained less intense indicating lower substrate input. The activity against Ubiquitin chains with a mixed K48 and K63 linkage was tested as well. Factor X showed no cleavage of these constructs. In case of the human recombinant Ubiquitin ligase A20 and USP7 and the Nedd8 specific NEDP1 no breakdown products could be observed but the bands with a molecular weight of 49 kDa, indicating di-Ubiquitin, are significantly weaker.

3 DISCUSSION

As during this work several aspects of SUMOylation were investigated, they are discussed separately in the following part. Later on, a conclusion gives an overview and integration of all achievements.

3.1 *In vitro* SUMOylation assays – a handy technique

In this work an *in vitro* SUMOylation assay system was established based on plant recombinant proteins that allows quick analysis of various proteins for their *in vitro* modification with different SUMO isoforms.

It turned out to be highly difficult to express and purify the *Arabidopsis* SUMO conjugating enzyme (SCE) in a stably active form from *E. coli*. Nevertheless it might be worth the effort, because the comparison of the plant recombinant enzyme to human recombinant SCE, which is commercially available, indicates that the *Arabidopsis* protein can utilize the SUMO isoform 3 with higher efficiency (Figure 2.1). Still, for SUMO1 such a difference was not detected. Due to this observation and the fact that the production of stably active plant recombinant SAE and SCE is quite challenging, commercially available human recombinant enzymes (BostonBiochem) were used in some subsequent experiments. Maybe human recombinant proteins have a lesser ability to use plant SUMO3, because this SUMO isoform differs rather significantly from the known mammalian SUMO variants. In contrast to this, *Arabidopsis* SUMO1 and SUMO2 show higher similarity to human SUMO2 and SUMO3, not only in their sequence, but also biologically in the formation of SUMO conjugates after stress treatment (Dohmen, 2004; Kurepa *et al*., 2003; Novatchkova *et al*., 2005). It might therefore be possible

that human recombinant enzymes can utilize *Arabidopsis* SUMO1 and SUMO2 with moderate efficiency, while the more distinct isoforms like SUMO3 are conjugated to a lesser extent.

The ability of the different SUMO moieties to form chains *in vitro* was analyzed as shown in Figure 2.2. SUMO1 and SUMO1 Q90A, in which a residue at position -4 from the carboxyl terminus is mutated, form chains in an identical pattern and to a similar extent. The differences in band intensity in the shown experiment are easily explained by the unequal amount of SUMO1 versus SUMO1 Q90A protein used. Both varieties form constructs with a molecular weight according to tetra-SUMO chains. In contrast to this, chain formation of SUMO3 and SUMO5 leads to only a single higher migrating band that agrees with a di-SUMO conjugate.

The ability of SUMO1 and SUMO1 Q90A to form chains with high efficiency might be due to lysine residues at position nine and ten in both proteins. These amino acids are conserved in SUMO2, where the second lysine at position ten is part of a typical SUMOylation consensus motif, and Colby *et al*. demonstrated chain formation of SUMO2 via those residues (Colby *et al*., 2006). SUMO3 has a lysine and a proline residue at this position, which might lead to weaker *in vitro* chain formation, because the lysine moiety is no longer part of a canonical consensus motif.

SUMO5 has at position nine and ten a serine and a proline, respectively. These residues might constrain chain formation and in case of the *in vitro* experiment, it cannot be ruled out that not only SUMO chains but other conjugates are detected that are stable during the gel electrophoresis under denaturing conditions. For example, stable SIZ-dependent SUMOylation of the SCE or the SIZ1 ligase itself was recently observed during *in vitro* SUMOylation assays with *Arabidopsis* proteins (Garcia-Dominguez *et al*., 2008). For the yeast and mammalian ortholog of the SCE, SUMOylation was reported as well, and it cannot be ruled out that

conjugates of SUMO and the conjugating enzyme are detected (Knipscheer *et al*., 2008).

The *in vitro* chain formation of various SUMO isoforms was also used to analyze the impact of the different enzymes. Comparison of the two different subunits of the activating enzyme, SAE1a and SAE1b, showed that the latter had a higher activity during *in vitro* experiments. Due to this, the SAE1b isoform was used later on.

It was also demonstrated that addition of a functional fragment of the SUMO ligase SIZ1 increases *in vitro* SUMOylation or SUMO chain formation and therefore this protein was added during *in vitro* SUMOylation experiments. SIZ1 might enhance the SUMOylation of different proteins *in vitro*, because *in vivo* a role for SIZ1 in many different pathways was shown, indicating a broad variety of substrates for this ligase (Catala *et al*., 2007; Jin *et al*., 2008). Low substrate selectivity was also detected for related yeast ligases of the SIZ/PIAS family, leading to the assumption that SIZ ligases can modify different substrates (Catala *et al*., 2007; Jin *et al*., 2008a; Reindle *et al*., 2006).

Different tags, commonly used for protein purification from *E. coli*, were monitored for their *in vitro* SUMOylation with SUMO1 (Figure 2.3). As SUMO1 is the most promiscuous of the tested SUMO isoforms, the analysis of this isoform was considered sufficient to gain information about the potential SUMOylation of tags. Neither the Flag tag, which was present in a fusion to the (*in vitro*) non-SUMOylatable protein UBC27 nor the GST-S construct, were modified. A combination of the three tags, the GST-S-Flag, was modified by SUMO1 *in vitro*. As only a single higher molecular weight band occurred, it can be assumed that this construct is mono-SUMOylated at a single residue. Mutation of a likely SUMOylation site close to the carboxyl terminus of the GST moiety, creating the GST (K217R)-S-Flag, did not abolish SUMOylation. Therefore it was assumed that a Lysine residue within the S peptide, which is part of a high probability SUMO

consensus motif might be SUMOylated. A combination of the GST(K217R) moiety and the Flag peptide lacking the S peptide was analyzed. The GST(K217R)-Flag construct showed no modification by SUMO1, indicating that a residue within the S peptide is likely to be SUMOylated. The modification seems to be context specific, because the GST-S protein remained unmodified. It can be hypothesized that the GST-S construct was not SUMOylated because the high probability SUMOylation site of the S peptide lies rather close to the carboxyl terminus of this protein. This might lead to a decreased interaction of the SUMOylating enzymes with the SUMO consensus motif.

The finding that the fusion protein Hap2a-GST could not be modified by any tested SUMO isoform during later experiments, strengthens the idea that the GST moiety itself is not SUMOylated (Figure 2.3). Therefore GST or Flag fusion proteins were used in subsequent experiments.

3.1.1 *In vitro* SUMOylation of different substrates

The *in vitro* SUMOylation system developed in this work was used to analyze the modification of various proteins, and to verify the results from Dr. Ruchika Budhiraja, who identified several potential *in vivo* SUMO targets. The results of these experiments are listed in Table 2.1 and in Figure 2.4.

Many of the identified SUMO substrates are proteins involved in either RNA-dependent or in DNA- or chromatin-related processes. This is concordant with the data for other organisms such as yeast or mammals, for which it was shown that many proteins from these pathways are modified by SUMO (Dohmen, 2004; Gill, 2005; Hay, 2005; Watts, 2004).

The efficiency of *in vitro* SUMOylation differed for the tested SUMO isoforms. Most proteins could be modified by SUMO1 and the mutant variety SUMO1 Q90A, which is lacking a conserved glutamine residue at position -4 from

the carboxyl terminus but is otherwise identical to SUMO1. A small subset of proteins was SUMOylated by SUMO3, but proteins modified with SUMO5 were not observed *in vitro*.

This is similar to the observations for the *in vitro* chain formation and might have different reasons. On the one hand only the b isoform of the activating enzyme was used, and this protein might not be able to activate SUMO3 and SUMO5 with the same efficiency as SUMO1. On the other hand, the same can be true for the added functional fragment of the SUMO ligase SIZ1, which might interact less efficiently with conjugates of the SCE and either SUMO3 or SUMO5.

The activity of SUMO1 and its mutant version SUMO1 Q90A agrees with the observation that SUMO1 and SUMO2 conjugates of different sizes increase strongly in *Arabidopsis* after heat shock or other stress treatments, leading to the assumption that these SUMOs have a broad range of substrates and can be quickly transferred onto targets (Kurepa *et al*., 2003). For SUMO3 and SUMO5 an accumulation of conjugates after heat shock was not observed and their abundance is far lower, so that they might have only few substrates or might modify targets only under very distinct conditions.

In case of modification by SUMO1 or SUMO1 Q90A, respectively, all proteins that contained high probability SUMOylation consensus motifs were modified. In these cases, the SUMOylation might proceed efficiently with SCE alone, which can interact directly with consensus sequences. Nevertheless, proteins such as TAF7, which lacked a consensus motif, could be SUMOylated as well. Interestingly, all substrates without high probability consensus motif contained several SUMO interacting motifs that may permit the interaction of the SCE-SUMO conjugate with the substrate via binding to the SUMO moiety. The SIZ1 ligase might also be necessary for modification of substrates without consensus sequence, but this was not further investigated.

The low efficiency for *in vitro* SUMOylation by SUMO3 and SUMO5 might occur, because some of the tested substrates are actually no targets of these isoforms.

However, candidate proteins that were identified as targets of SUMO5 *in vivo*, such as TAF7, could not be modified with this SUMO moiety *in vitro*. So it seems to be more likely that the lack of SUMO5 conjugation is rather due to an inability of the tested enzymes to efficiently interact with this SUMO isoform.

SUMOylation with SUMO3 was far less effective than modification by SUMO1 or SUMO1 Q90A, but could be observed for the proteins TAF7, SVP and NAF. It is striking that these three proteins contain several SIM motifs but not necessarily a high probability SUMOylation consensus motif. Their SUMOylation might be facilitated by interaction of the SIM motifs with the second beta sheet of the SUMO moiety, because the SUMO3 sequence is rather conserved in this structural motif except for one amino acid exchange that supposedly does not alter the interaction with hydrophobic amino acids, and it can safely be assumed that the change does not lead to a different secondary structure (Novatchkova *et al*., 2004). SUMO3 modification led only to one slower migrating band, indicating mono-SUMOylation by a single SUMO moiety. This agrees with the low ability of this SUMO isoform to form poly-SUMO chains. In contrast to this, the occurrence of several bands during SUMOylation by SUMO1 or SUMO1 Q90A might be partly due to modification with SUMO chains and is therefore not necessarily the result of SUMOylation of different residues within the substrate. However, in case of Rtx3, a protein with many SUMOylation consensus sites, a multitude of higher molecular weight bands occurred, indicating SUMOylation of several Lysine residues within the same substrate protein.

3.1.2 Determination of SUMOylation sites

After our attempts to detect the SUMOylation sites of several proteins by mass spectrometry failed, we decided to take a different approach for the TAF7 protein that is part of the TFIID complex, a basic transcription factor in RNA-Polymerase II dependent transcription.

Several mutant varieties of TAF7 were created, in which the lysine residue that is the potential acceptor of the SUMO moiety is mutated to arginine that cannot be SUMOylated.

As shown in Figure 2.5, the mutation of single lysine residues or the double exchange of the two neighbouring amino acids K423 and K424 did not abolish SUMOylation with SUMO1. Still the experiment indicated less efficient modification of TAF7 varieties lacking the lysine residues K373, K423, K424 or K435 close to the C-terminus. Therefore the according triple and quadruple mutants were generated and analyzed for their *in vitro* SUMOylation. The proteins TAF7(K373,423,424R) and TAF7(K373,423,424,435R) were modified with the same efficiency as wild type protein. For TAF7(K423,424,435R), no SUMOylation was detected, but because of the strong modification of the quadruple mutant TAF7(K373,423,424,435R), it has to be assumed that the failure to detect modified triple mutant protein in this particular experiment is not representative. In this experiment, less TAF7(423,424,435R) substrate was used, so that the SUMOylation of this protein might be below the threshold of detection.

The combined results with mutant TAF7 proteins indicate that the previously observed weaker higher molecular weight bands during SUMOylation of the TAF7 varieties mutated in lysine residues close to the carboxyl terminus are more likely due to unequal staining of the according part of the Western blot, than to differences in the SUMOylation reaction. For these experiments it cannot be ruled out completely that the use of human recombinant conjugating enzyme during these experiments might influence the results, but this is highly unlikely because the TAF7 lacks a SUMO consensus motif that would interact directly with this enzyme. In addition, the *Arabidopsis* SUMO ligase SIZ1 was added, which most likely facilitates the modification of proteins lacking a consensus sequence. The findings should therefore not differ from those obtainable with plant recombinant SCE.

It is striking that TAF7 modification does not differ for mutated proteins. Nevertheless it may not be forgotten that not all lysine residues were mutated. A subset was chosen regarding preliminary mass spectrometry results, indicating the mutated amino acids as probable SUMOylation sites. Therefore it cannot be ruled out that the mutated amino acids were not chosen carefully enough. This would have to be proven by subsequent experiments, in which the other lysine residues are mutated as well. Another option is that SUMOylation is not restricted to a single residue within the protein, but can switch between different amino acids.

SUMOylation of unaltered TAF7 led to a single distinct higher molecular weight band that indicates mono-SUMOylation at a single residue. Modification of several lysine residues within the proteins would rather result in a pattern with several less strong bands as found for the target Rtx3 which has multiple high probability SUMOylation sites.

The analysis of TAF7 demonstrated that the *in vitro* SUMOylation may not be restricted to a certain residue within this protein, but seems to occur at multiple sites. Nevertheless the overall SUMOylation does not exceed one SUMO moiety. Maybe structural restrictions prohibit the attachment of more than one SUMO protein. Whether this happens, because second site SUMOylation is generally rare or because SUMOylation triggers conformational changes that inhibit further modification, remains speculative.

In *Arabidopsis* SUMOylation of TAF7 might be involved in transcriptional control. TAFs, TBP-association factors, are able to interact with a TATA-box binding protein to form the basic TFIID transcription factor. This protein complex is involved in RNA Polymerase II dependent transcription, and TAFs confer TFIID ability to recognize TATA-less promoters (Thomas & Chiang, 2006). Modification by SUMO1 was demonstrated for human TAF5 and TAF12 (Boyer-Guittaut *et al*., 2005). While TAF12 SUMOylation had no obvious effect, modification of TAF5 interfered with TFIID binding to promoter regions indicating a regulatory role for

SUMOylation in TFIID mediated transcription. Additionally, a role for SUMOylation during assembly and disassembly of protein complexes in general was discussed, and the TFIID consists of several subunits that can exist in different combinations.

A similar role for TAF SUMOylation in *Arabidopsis* has not been demonstrated yet, but seems likely regarding our results for the TAF7 protein.

3.2 SUMOylation by SUMO1 Q90A

Most SUMO isoforms carry a glutamine residue at position -4 from the C-terminus. This is conserved throughout several organisms, but the natural isoforms SUMO3 and SUMO5 have a leucine and a methionine at this position, respectively, nevertheless they are expressed and conjugated *in vivo* (Novatchkova *et al*., 2004; Saracco *et al*., 2007).

We investigated the role of this residue further. Therefore Dr. Ruchika Budhiraja and Dr. Andreas Bachmair generated transgenic plants, which over-express SUMO1 variants, in which this residue was exchanged. The results of Dr. Budhiraja suggested similar effects, when this residue was mutated to leucine or to alanine. The amino acid exchange led to growth retardation and premature leaf senescence, which resulted in plant death if the construct was constitutively expressed. Under an inducible promoter, this phenotype was less severe and an accumulation of SUMO conjugates for SUMO1 Q90A was observed compared to SUMO1 conjugates in transgenic plants.

Therefore I investigated the role of the Glutamine residue situated at position -4 from the C-terminus further.

During *in vitro* SUMOylation assays, no difference between SUMO1 wild type and the mutant variety SUMO1 Q90A was observed. Both SUMO variants modify the

same substrate to a similar extent and with an identical pattern of higher molecular weight bands (Figure 2.4). This is most likely due to modification of identical sites within the protein, because otherwise the migration and pattern of SUMOylated species would differ.

In Figure 2.7, it was also demonstrated that SUMO1 Q90A is incorporated into slower migrating bands after heat shock treatment of seedlings. The accumulation of conjugates after diverse stress treatments is a typical feature of SUMO1 as shown by Richard Vierstra and his co-workers (Kurepa *et al.*, 2003). So it seems that both, wild type SUMO1 and SUMO1 Q90A, do not differ in this respect. However, the natural isoforms SUMO3 and SUMO5, which lack the conserved glutamine at position -4, do not form more conjugates in response to stress. The lack of SUMO conjugates with SUMO1 Q90A prior to stress treatment is rather surprising, because SUMO1 conjugates are also found in unstressed plants. Maybe the amount of conjugates is too low for detection with the used antibody. Apart from that, the untagged natural SUMO1 that is also present in the seedlings could be preferred to the tagged mutant SUMO1 Q90A during conjugate formation. Only under stress conditions, when the amount of natural SUMO moieties might be insufficient, the mutant SUMO1 Q90A conjugates increase to a detectable level. Nevertheless it can be assumed that both, SUMO1 and SUMO1 Q90A, do not differ in their conjugation properties.

3.2.1 De-SUMOylation of SUMO1 Q90A

After it became clear that the SUMO1 Q90A variant does not seem to differ in conjugation from the SUMO1 wild type, its features during *in vitro* de-SUMOylation were analyzed. As shown in Figure 2.8, both SUMO1-NAF and SUMO1 Q90A-NAF conjugates are cleaved by a functional fragment of the SUMO protease ESD4 during a time course of one hour, but degradation of the SUMO1 Q90A conjugate is far slower and after sixty minutes SUMO1 Q90A-NAF is still

observed, while the SUMO1 conjugate was no longer detectable after 15 min incubation with ESD4. The SUMO1 Q90A conjugate is therefore degraded at least four times slower than the respective SUMO1 wild type conjugate. A similar experiment was repeated with less protease and secondary antibody coupled to IR dye, which allowed quantification of the conjugates. Again, SUMO1 Q90A conjugates showed a higher resistance against the SUMO protease ESD4. After 30 min, 70% of the employed SUMO1-NAF was cleaved while SUMO1 Q90A de-conjugation was not detected.

The increased stability of SUMO1 Q90A conjugates against ESD4 *in vivo* agrees with the results of Dr. Budhiraja. She postulated that plants over-expressing SUMO1 Q90A accumulate more SUMO conjugates than plants producing SUMO1 under an inducible promoter (Budhiraja, 2005). *In vitro* only a single protease, ESD4, was tested for its ability to cleave conjugates with SUMO1 Q90A, but it seems that this enzyme is a major SUMO protease in *Arabidopsis* nuclei, and up to now no other *Arabidopsis* SUMO protease with a similar effect on plant development was described (Colby *et al*., 2006; Murtas *et al*., 2003; Reeves *et al*., 2002). Therefore the resistance of SUMO1 Q90A conjugates against the functional fragment of ESD4 might be sufficient to explain the increased stability of SUMO1 Q90A conjugates *in vivo*.

The model that the amino acid exchange in SUMO1 Q90A can stabilize conjugates is supported by structural data from Lima and co-workers, who demonstrated that SUMO proteases interact with this region of the SUMO moiety during cleavage of SUMO conjugates and that residues C-terminal of the Gly-Gly motif are important for protease specificity towards different SUMO isoforms (Mossessova & Lima, 2000; Reverter & Lima, 2004). In our experiments, only mature SUMO moieties were expressed either as recombinant proteins or in *Arabidopsis*. So we did not address the question whether differences occur only during de-conjugation of substrates or might affect maturation of the SUMO precursor as well. It is very

likely that the residues in close proximity to the di-glycine motif affect the maturation rate of different SUMO isoforms *in vivo*.

Our observations for the variant SUMO1 Q90A imply also an increased stability of SUMO3 and SUMO5 conjugates, which might coincide with slower maturation rates. This could explain, why under stress conditions a quick increase of SUMO1 and SUMO2 conjugates, but not off SUMO3 or 5, is observed.

As SUMO1 and SUMO1 Q90A showed no differences in conjugation, but the SUMO1 Q90A conjugates are more stable, the mutant variety might turn out to be a valuable tool to detect SUMO1 substrates. The modified proteins should not differ *in vitro* and likely *in vivo* for both SUMO1 variants. Nevertheless, the increased stability of SUMO1 Q90A conjugates allows easier purification and handling of substrates during subsequent experiments.

3.3 PIAS-like SUMO proteases in *Arabidopsis*

In this work, not only *in vitro* experiments were conducted, but several plants mutated in enzymes of the SUMOylation cycle were analyzed as well. One class of proteins, whose role in plants was further studied, are the SUMO ligases of the SIZ/PIAS family.

Arabidopsis has three genes potentially coding for SUMO ligases of the SIZ/PIAS family, SIZ1 (At5g60410), PIAS-Like1 (At1g08910) and PIAS-Like2 (At5g41580) (Novatchkova *et al*., 2004). These types of proteins are known SUMO ligases in yeast and animals and are characterized by their SP-RING also called zf-MIZ domain (Johnson & Gupta, 2001). They show similarity to Ubiquitin ligases of the RING type and can interact with the SCE-SUMO conjugate via their SP-RING domain.

While the role of PIAS-Like1 (PIL1) and PIAS-Like2 (PIL2) remains unknown, SIZ1 is already well described. This SUMO ligase plays a regulatory role in the response to various stresses like cold, drought or phosphate starvation and is involved in regulation of flowering time and salicylic acid signalling (Catala *et al*., 2007; Jin *et al*., 2008b; Lee *et al*., 2007; Miura *et al*., 2007; Miura *et al*., 2005; Yoo *et al*., 2006). SIZ1 seems to be able to SUMOylate the transcription factors ICE1, PHR1, FLD and the bromo domain containing protein GTE3 (Garcia-Dominguez *et al*., 2008; Jin *et al*., 2008b; Miura *et al*., 2007; Miura *et al*., 2005).

Whereas the involvement of SIZ1 in many different developmental traits and responses to biotic and abiotic factors is clear, nothing is yet known about the role of the other two members of this protein family. We therefore investigated the two potential SUMO ligases PIAS-Like1 (PIL1) and PIAS-Like2 (PIL2).

3.3.1 PIAS-Like1 and PIAS-Like2 differ from SIZ1 in their domain structure

As shown in Figure 2.9, the domain structure of SIZ1 differs from PIL1 and PIL2. All three proteins contain a zf-MIZ domain that shows similarity to the U-box of Ubiquitin ligases and is responsible for SUMO ligase activity. Nevertheless, in SIZ1, two additional domains are found: a SAP domain and a PHD finger. The SAP domain is involved in interactions with RNA and DNA and is often found in nuclear and cytoplasmic proteins (Aravind & Koonin, 2000; Iida *et al*., 2006). The PHD is a C4HC3 Zn-finger-like motif with similarities to the RING finger. It is typically found in nuclear proteins that are involved in chromatin-mediated transcriptional regulation, and interactions of this domain with methylated histone H3 were shown. The occurrence of those regulatory domains in SIZ1 might explain its various regulatory roles in different pathways.

Recently it was also demonstrated that the PHD finger can interact with the SUMO conjugating enzyme and contributes together with the SP-RING (zf-MIZ) domain to the SUMO ligase activity (Garcia-Dominguez *et al*., 2008).

Furthermore, SIZ1 contains several high probability SUMOylation consensus motifs that can explain the observed SUMO modification of this enzyme during *in vitro* experiments (Garcia-Dominguez *et al*., 2008). As those motifs are absent in PIL1 and PIL2, their auto-SUMOylation seems rather unlikely.

In contrast to SIZ1, PIL1 and PIL2 have only a zf-MIZ domain, which might enable these proteins to function as SUMO ligases. In PIL1 the SP-RING is lacking two conserved cysteine residues and a proline close to the C terminus of the domain. It is therefore unclear whether this protein contains a functional zf-MIZ finger, because it was suggested that those residues, which are conserved throughout zf-MIZ domains of several organisms, are integral part of the RING motif and coordinate the co-factor zinc (Cheng *et al*., 2006). To investigate whether PIL1 and PIL2 have indeed SUMO ligase function, the amount of SUMO conjugates in the according mutant plants was analyzed.

3.3.2 SUMO conjugates of SUMO ligase mutants

To analyze the potential role of the PIL proteins as SUMO ligases, the amount of SUMO1 conjugates of different SUMO ligase mutants was analyzed, because this SUMO isoform is highly expressed and seems to target various proteins. It is likely that the used antibody (ABCAM) might interact with the nearly identical SUMO2 as well (Kurepa *et al*., 2003).

As shown in Figure 2.10, the wild type plants have a relatively low level of SUMO1 conjugates during normal growth conditions that increases strongly after heat shock treatment as described before by Richard Vierstra and his co-workers (Kurepa *et al*., 2003). Interestingly, although for seedlings significant SUMO1 expression was assumed, free SUMO was detected in no case, which might be due

to a relatively low expression level of SUMO in the tested plants, (Saracco *et al*., 2007).

The tested *siz1* mutants have a decreased amount of SUMO conjugates under standard conditions compared to wild type, and do not show an increase of SUMO modified proteins after heat shock treatment. The *siz1a* line, which has in general a stronger growth defect than mutants with the *siz1f* allele, shows a lower level of SUMO1 conjugates also upon heat shock.

This is consistent with previous work that links the SIZ1 SUMO ligase to various stress-related pathways like drought tolerance, phosphate starvation or cold adaptation (Catala *et al*., 2007; Miura *et al*., 2007; Miura *et al*., 2005; Yoo *et al*., 2006). SIZ1 also seems to play a role in the response to biotic stresses (Lee *et al*., 2007) and so far all substrates targeted by this ligase seemed to be modified by SUMO1. SIZ1 is also able to increase SUMO1 modification during *in vitro* SUMOylation assays as reported in the literature and demonstrated in this work (Garcia-Dominguez *et al*., 2008; Jin *et al*., 2008; Miura *et al*., 2007; Miura *et al*., 2005). These results strongly indicate a role for SIZ1 in modification of different proteins by SUMO1 and maybe SUMO2 and also a function in the general increase of SUMO conjugates during stress response.

The results from the *pil* mutants diverge, although the two potential SUMO ligases are highly similar. In case of the *pil1* mutant, the overall amount of SUMO1 modified proteins is slightly less compared to wild type under standard conditions and does not increase after heat shock. In contrast to this, the *pil2* mutant has an increased level of SUMO conjugates prior to and after stress treatment. The according double mutants show slightly less SUMO conjugates under both tested conditions compared to the wild type.

The slightly lower level of SUMO1 conjugates in the *pil1* mutant seedling is surprising, because this transgenic plant line has no obvious phenotype. This contrasts with the *siz1* mutants that display a similarly disturbed SUMOylation pattern, but have a severe growth defect. As only a single plant was analyzed for

each tested condition, this observation might be due to variation among individuals. Therefore it cannot be ruled out that the weak difference of *pil1* compared to wild type is indeed due to a SUMO ligase function of PIL1. With respect to the aberrations in the SP-RING of PIL1 it is possible that this domain is not functional and that PIL1 cannot act as a SUMO ligase by itself. However, it might be a subunit in a SUMO ligase complex, or regulate other SUMO ligases like SIZ1.

The *pil2* mutants have an increased amount of SUMO1 modified proteins under standard conditions and a level comparable to those of wild type plants after stress treatment. Maybe the knockout of the PIL2 protein is stressful to plants at this developmental stage and leads to an accumulation of SUMO1 conjugates under normal conditions. The mutation seems to have no severe effect on growth, because adult plants do not have an obvious phenotype and cannot easily be distinguished from wild type.

Both tested lines of *pil1pil2* double mutants have less SUMO conjugates under standard and stress conditions compared to wild type in the experiment with horse radish peroxidase-coupled antibody, but the results of the Western blot with alkaline phosphatase-coupled secondary antibody do not confirm this observation. Nevertheless both experiments show a strongly reduced level of SUMO conjugates in *pil1pil2* under standard conditions compared to the *pil2* single mutant. The reduced level of SUMO modified proteins indicates a SUMO ligase function of at least one of the PIL proteins. The present conjugates might be formed by the prominent SUMO ligase SIZ1, which is present in *pil1pil2* mutants. The findings that the amount of SUMO conjugates is only slightly reduced and that the plants have no severe phenotype compared to *siz*, indicates only a minor role for PIL1 and PIL2 in *Arabidopsis* SUMO1 conjugation. The single mutants of the PIL ligases gave no clear results and it cannot be ruled out that these highly similar proteins are functionally redundant or might be co-regulated. In this case,

the knockout of PIL2 might lead to an up-regulation of PIL1 or SIZ1 that can explain the increase of SUMO1 conjugates under standard conditions.

Taken together, these experiments suggest a SUMO ligase function for the PILs, but it may not be forgotten that they were only performed with a single individual for each condition and have to be repeated with another set of biological replicates. It is also of great interest to verify the SUMO ligase function of PIL2 and PIL1 *in vitro* by creating recombinant PIL proteins for subsequent SUMOylation assays. As the Western blot data indicate a minor role for PIL1 and PIL2 in SUMO1 conjugation, their relevance for SUMO3 and SUMO5 conjugation should be analyzed, because so far no role of the third PIAS family member in *Arabidopsis*, the SIZ1, on SUMO3 and SUMO5 modification has been demonstrated, but these two SUMO isoforms seem to be conjugated nevertheless (Budhiraja, 2005).

3.3.3 Phenotypic analysis of *pil* mutant plants

The growth and the development of *pil1* and *pil2* single and *pil1pil2* double mutants was analyzed but no difference from wild type under either long day or short day conditions was observed (Figure 2.11).

In contrast to this, the *siz1* mutants showed a strongly reduced growth and decreased apical dominance as already described in the literature (Catala *et al*., 2007; Miura *et al*., 2007; Miura *et al*., 2005; Yoo *et al*., 2006).

The flowering time of the different mutants was also monitored (Figure 2.12).

The *pil1pil2* plants did not differ significantly from wild type under long day (sixteen hours light per day) or extended short day conditions. Under these conditions, earlier flowering of *siz1a* and *siz1c* was observed, while the *siz1f* that has an overall weaker phenotype, did not differ from the wild type. Nevertheless, under a light regime with only eight hours light per day, the *siz1f* and the *pil1pil2* mutants flowered later compared to the wild type, but the *siz1a* and *siz1c* plants did not differ significantly compared to Columbia 0 plants. This observation is

rather striking, because in the literature early flowering for *siz1* knockout lines has been described (Jin *et al*., 2008; Miura *et al*., 2005) and the later flowering of *siz1f* could not be confirmed in another experiment under short day conditions (Figure 2.17).

It has to be kept in mind that only a single experiment under short day conditions was performed and that the standard deviation is rather high and adds up to a quarter of the total leaf number (for several lines more than twenty leaves). Additionally, the two *pil1pil2* lines differ in their cauline leaf number. While the line *pil1cpil2a* has less cauline leaves compared to Columbia, *pil1apil2b* does not differ significantly from the wild type. Concerning the observation that the *siz1* lines differ in the discussed experiment from the known literature and that the later flowering of *siz1f* was not observed in another experiment, it has to be assumed that the results of the flowering time experiment under short day conditions are inconclusive, and that this experiment has to be repeated to give clearer information about *pil1pil2*. However, it can be concluded from these experiments that the PIL SUMO ligases have a less important function in flowering time control than SIZ1.

Generally, our results indicated only a minor role for PIL1 and PIL2 in SUMO conjugation under the tested conditions, and it has to be assumed that SIZ1 is the major SUMO ligase in *Arabidopsis* that is involved in a broad variety of different pathways.

In contrast, our cooperation partner Dr. Holger Hesse from the MPI for Plant Physiology, Golm, Germany, analyzed the metabolite content of the tested *pil* mutants, because he investigates a sulfure transporter, which is likely to be regulated by SUMOylation (Holger Hesse, personal communication). The preliminary data obtained in his laboratory indicate altered levels of different metabolites in the *pil1pil2* double mutants compared to *siz1* mutants or wildtype. For example, an upregulation of Serine and a downregulation of different sulfur

transporters were observed (data not shown). The results of Dr. Hesse imply a role for PIL1 and PIL2 in regulation of sulfur metabolism and uptake. For SIZ1 a role in the primary metabolism of *Arabidopsis* was already demonstrated by showing that *siz1* plants are more sensitive to phosphate starvation compared to wild type (Miura *et al*., 2005). It is therefore not unlikely that other pathways are also regulated via SUMOylation. An involvement of the PIL proteins in sulfur metabolism might not lead to an obvious phenotype under the conditions tested by us, because sulfur starvation or boosted sulfate levels in the media were not tested. Therefore, it would be of great interest to see the results of sulfur starvation experiments that are currently carried out by our cooperation partners in Golm, to verify a regulatory role of PIL1 and PIL2 in sulfur metabolism.

3.4 The SUMO protease EL1

Not only SUMO ligases, but also SUMO proteases were analyzed in this work.
The *Arabidopsis* genome encodes several SUMO proteases. One of them, Early in Short Days 4 (ESD4, At4g15880), plays an important role in control of flowering time by regulation of the floral repressor FLC. Mutants in this SUMO protease flower extremely early under short days (Murtas *et al*., 2003; Reeves *et al*., 2002).
In this work, the closest homolog of ESD4, the SUMO protease Early in Short Days 4-Like 1 (EL1, also called Ulp1a,At3g06910) was further investigated to analyze its role in plant development with particular emphasis on flowering time control.

3.4.1 Domain structure of EL1

The domain structure of El1 was compared to its two closest homologs in *Arabidopsis*, ESD4 and Ulp1b (At4g00690) as depicted in Figure 2.13. All three

proteins contain a Peptidase_C48 domain. This feature is typical for cysteine proteases with activity against SUMO and SUMO conjugates and SUMO protease activity has already been reported for ESD4 and EL1 (Colby *et al*., 2006; Murtas *et al*., 2003; Reeves *et al*., 2002).

Although the peptidase domain of ESD4 and Ulp1b is more similar compared to EL1, overall similarity is higher between ESD4 and EL1. Both, ESD4 and EL1 contain an amino terminal extension that is absent from the smaller Ulp1b.

No domains or obvious structures were found in these areas with PFAM webservice (http://pfam.sanger.ac.uk), but the analysis with the SUMOplot tool and the search for potential SUMO interaction motifs, revealed some differences in the amino terminal regions of ESD4 and EL1. Both share a potential SUMOylation motif (TD**VKKG**EN, VAS**LKNG**SL), but another high probability SUMOylation site is exclusively found in ESD4 (PD**AKAP**LR).

Strinkingly, in ESD4 and Ulp1b only a single potential SUMO interaction motif is found on the amino terminal site of the catalytic domain, while in EL1 four of these motifs occur in the amino terminal region before the Peptidase_C48 domain.

The multiplicity of potential SUMO interaction sites in EL1 indicates an affinity for poly-SUMOylated substrates, because they could allow interaction with poly-SUMO chains. A similar mechanism was already demonstrated for a class of SUMO targeted Ubiquitin ligases (Stubls), which contain several SIM domains and preferably ubiquitylate poly-SUMOylated targets (Lallemand-Breitenbach *et al*., 2008; Perry *et al*., 2008; Prudden *et al*., 2007; Tatham *et al*., 2008; Uzunova *et al*., 2007; Weisshaar *et al*., 2008).

Therefore it seems likely that the amino terminal SUMO interaction motifs of EL1, which are absent in ESD4, play a role in substrate selectivity of these two similar proteins.

3.4.2 Localization of EL1

After attempts to express GFP-tagged EL1 in *Arabidopsis* failed, transient expression of EL1 fused to GFP in *Nicotiana benthamiana* was used. GFP fusions to both the carboxyl- and amino-terminal end of EL1 were absent from the nucleus and the nuclear membrane, but could be detected in the cytoplasm around the nucleus (Figure 2.14). Often vesicle-like structures were observed, which might be attached to cytoplasmic strands. The observation of aggregates in case of GFP-EL1 is most likely due to the strong overexpression of the construct in tobacco cells.
The localization of EL1 in the cytoplasm and in vesicular structures differs completely from ESD4, which was found in the nucleus, predominantly at the nuclear periphery, maybe associated to the nuclear inner envelope. It cannot be ruled out that the observed vesicles are, like aggregates, an artefact of over-expression. It might nonetheless be interesting to analyze a connection to the Endoplasmatic Reticulum or the Golgi apparatus, two organelles involved in membrane trafficking.

3.4.3 Phenotypic analysis of *el1* mutant plants

The analysis of the growth phenotype of *el1* mutant plants revealed that *el1* mutants do not differ significantly in height and number of side shoots from the wild type control (Figure 2.15). In case of *el1a*, which is in the background of the ecotype Wassilewskija, thinner stems compared to the respective wild type were observed. This observation was confirmed by an Astralblue/Safranin T and a Safranin T stain shown in Figure 2.16. Both staining methods showed that in *el1* stems the ratio of vascular tissue to mesophyll is increased in comparison to (thicker) wild type main shoots at the same developmental stage or to side shoots of the wild type plant with a similar diameter. In contrast to the *el1a* in Wassilewskija wild type, the other two lines in the background of Columbia did

not differ in their stem thickness from the respective wild type. It therefore seems that the thinner stems of *el1a* are dependent on the genetic background and not due to a general effect of EL1 independent of the ecotype.
The flowering time of the different *el1* mutants was also monitored (Figure 2.17). The number of rosette leaves during the time of the opening of the first flower did not differ significantly from the wild type in case of all tested *el1* mutant lines. An increased number of cauline leaves for *el1a* was observed in a single experiment under a sixteen hours light regime, but could not be confirmed in a second experiment with long day conditions. It seems therefore safe to assume that EL1 plays no role in control of flowering time.

It can be summarized that ESD4 and EL1 differ clearly in their localization and in the phenotype of the respective mutants, although the proteins are highly similar. Therefore, it has to be concluded that both SUMO proteases differ in their substrates. EL1 might have a distinct set of target proteins due to its cytoplasmic localization and its many SUMO interaction motifs.
The analysis of mutant plants showed only a weak effect of the *el1* mutation on plant growth and development, and indicated that at least in the ecotype Wassilewskija, EL1 might play a minor role in the differentiation of stems. In both the Columbia and the Wassilewskija background, EL1 seems to play no role in flowering time control. Therefore ESD4 seems to be the key SUMO protease of the Ulp1 type in *Arabidopsis.*

3.5 Early in Short Days 4 (ESD4), a SUMO protease involved in flowering time control

Early in Short Days 4 (ESD4, At4g15880) seems to be the key SUMO protease of the Ulp1 type in *Arabidopsis*. It has already been demonstrated that it regulates flowering time (Murtas *et al*., 2003; Reeves *et al*., 2002). ESD4 presumably controls the activity of the floral repressor FLC and mutants of this SUMO protease flower extremely early under short day conditions (Murtas *et al*., 2003; Reeves *et al*., 2002). ESD4 is localized in the nucleus, mainly at the inner side of the nuclear envelope and possesses a typical SUMO protease domain (Peptidase_C48 domain,Figure 2.13), and its SUMO protease activity has already been demonstrated (Murtas *et al*., 2003; Reeves *et al*., 2002).

In addition to the early flowering phenotype in short days, *esd4* plants differ also from wild type in their bushy growth and decreased apical dominance. As it is assumed that a similar growth defect of the *siz1* mutant is (partly) due to increased levels of salicylic acid (Catala *et al*., 2007; Lee *et al*., 2007), we investigated whether the growth phenotype of *esd4* is due to altered levels of this hormone as well. The *esd4* mutants were crossed to *sid2* mutants that are defective in salicylic acid biosynthesis and have a lower content of this phyto hormone. Introduction of the s*id2* allele into *esd4* plants should decrease the salicylic acid level in the offspring. As shown in Figure 2.18, the resulting *esd4sid2* double mutants showed a growth defect identical to the *esd4* single mutants. The heterozygous mutants *esd(het)* and *esd4(het)sid* had a less severe growth defect, compared to *esd(hom)* and *esd4(hom)sid.* Although this result is rather clear, a similar experiment is ongoing, in which the *esd4* mutants had been crossed to plants carrying the bacterial *nah*G gene coding for a salicylic acid hydroxylase, which reduces the level of salicylic acid in plants by degrading the hormone.

It seems that the growth defect of the *esd4* mutants and their decreased apical dominance are independent of salicylic acid signaling, in contrast to the salicylic acid dependence of a similar growth phenotype of for *siz1* mutants. Therefore, it has to be assumed that, although SIZ1 seems to be the major SUMO ligase in *Arabidopsis* and ESD4 the major SUMO protease, both enzymes act in different pathways regulating plant growth.

3.6 The bacterial effector protein Factor X

The plant pathogen *Xanthomonas campestris*, which attacks S*olanaceae* such as pepper or tomato, is able to inject so-called type III effectors into host cells (Gurlebeck *et al*., 2006). One of these virulence factors is XopD, a cysteine protease with a peptidase_C48 domain, which is characteristic for SUMO proteases (Chosed *et al*., 2007; Hotson *et al*., 2003; Kim *et al*., 2008). After delivery into the host cell, XopD is located in the nucleus and it is therefore likely to process nuclear targets. Its SUMO protease activity has been demonstrated both *in vivo* and *in vitro* (Chosed *et al*., 2007; Colby *et al*., 2006; Hotson *et al*., 2003).

Our cooperation partners Prof. Ulla Bonas and Robert Szczesny from the Martin-Luther-University in Halle identified Factor X, a *Xanthomonas* type III effector protein with similarity to XopD. Therefore, Factor X was tested for protease activity against protein modifiers. As the data of our cooperation partners about Factor X are not yet published, no further details can be revealed regarding the nature of Factor X.

3.6.1 *In vitro* protease activity of Factor X

The analysis of bacterial proteins for their *in vitro* protease activity against SUMO1 is rather difficult, because it was shown that those virulence factors might display nonspecific protease activity if the experimental settings are not chosen well, although the natural function of the proteins might be different. In case of the *Yersinia pestis* effector YopJ for example, it was first postulated that this protein is a SUMO protease (Orth *et al*., 2000). Later on, it turned out that YopJ has far higher activity as an acetyltransferase, and that the observed SUMO protease activity is likely restricted to the *in vitro* experiment (Mukherjee *et al*., 2006).

To test the *in vitro* protease activity of Factor X, the protein was produced and purified from *E. coli* as a His tag fusion (clone received from R. Szczesny). A mutant variety, in which the catalytic cysteine residue was exchanged to alanine, was used as a negative control. Its activity towards SUMO1, SUMO3 and Rub1 fusion proteins that were produced as recombinant proteins in our laboratory, as well as against the commercially available human recombinant Ubiquitin chains with either a K48 or a mixed linkage was tested in Figure 2.19.

Factor X was not able to cleave a SUMO1 or a SUMO3 fusion protein. A functional fragment of ESD4 that was produced in the same way and tested under identical conditions showed rather strong activity against both substrates. It can therefore be assumed that Factor X has indeed no *in vitro* SUMO protease activity and the lack of activity is not due to deficiencies in the experimental design.

Similar results were obtained for cleavage of Rub1, the *Arabidopsis* ortholog of Nedd8. Factor X was not able to cleave a Rub1 fusion protein while the recombinant human protease NEDP1 was able to cleave Rub1 in this fusion. The ability of the human protease to cleave a plant substrate shows that this protein modifier is highly conserved throughout the different kingdoms, and that in this *in vitro* experiment high protease activity can be achieved.

Finally, the activity of Factor X against Ubiquitin chains was analyzed. These substrates were not produced during this work, but obtained from BostonBiochem

as human recombinant proteins. Factor X was not able to cleave K48 chains or chains with mixed K48/K63 linkage. In case of K_{48} chains, a significant activity of NEDP1 and A20 was observed, while the result for recombinant human ubiquitin-specific protease USP7 is less clear. As in case of USP7 and K48 chains, a low amount of protein in these two lanes made interpretation difficult. Nevertheless, cleavage by NEDP1 and A20 is documented by occurrence of bands with lower molecular weight. This shows again that a strong protease activity was obtained, because NEDP1 is actually a Nedd8 (Rub1) protease in contrast to the Ubiquitin-specific A20 and USP7.

The results for Ubiquitin chains with a mixed linkage are less clear. Again, Factor X was not able to process the substrate. In case of the human recombinant control proteases NEDP1, A20 and USP7, no additional bands appeared that indicated cleavage, but the bands with a molecular weight of approximately 49 kDa that indicate tetra-Ubiquitin are significantly weaker. It might therefore be that these proteases did indeed cleave the tetra-Ubiquitin constructs but that it was not possible to detect the released mono-Ubiquitin.

Only human recombinant Ubiquitin chains were tested, but nevertheless it can be assumed that Factor X has no activity against *Arabidopsis* Ubiquitin fusions, because Ubiquitin is one of the most conserved proteins throughout all eukaryotic kingdoms.

In summary, Factor X has - at least *in vitro* - no protease activity against the tested protein modifiers, while the recombinant control proteases were active under identical conditions. These results agree with the observation that Factor X has also no *in vivo* SUMO protease activity (R. Szczesny, personal communication). Weak protease activity of recombinant Factor X that was observed before (R. Szczesny, personal communication) might therefore be the result of contamination with bacterial proteins.

4 CONCLUSION

In this work, several aspects of *Arabidopsis* SUMOylation were discussed:

- An *in vitro* SUMOylation system based on plant recombinant proteins was developed, which can be used to study the modification of various potential substrates. At a large scale, *in vitro* SUMOylation allows purification of SUMOylated proteins for subsequent experiments. This method can also be applied to further investigate the different enzymes involved in SUMOylation as well as the differences of the SUMO isoforms.

- A mutant variety of SUMO1, the SUMO1 Q90A was analyzed, in which a conserved glutamine at position -4 from the carboxyl terminus is changed to alanine. *In vitro* experiments revealed that this variant leads to conjugates with higher resistance against the SUMO protease ESD4, but is identical to SUMO1 concerning modification of substrates and SUMO chain formation. SUMO1 Q90A is also incorporated into conjugates after heat shock similar to SUMO1 wild type. SUMO1 Q90A can therefore be used to enrich SUMO1 conjugates in plants: It will presumably lead to an identical subset of modified proteins but give more stable conjugates. The insight that an alteration of the conserved glutamine close to the carboxyl terminus increases conjugate stability leads also to the assumption that the natural isoforms SUMO3 and SUMO5, which have leucine and methionine, respectively, at this position, form more robust conjugates *in vivo*.

- The PIAS-Like1 and PIAS-LIKE2 proteins, two potential SUMO ligases of the SIZ/PIAS type, were investigated as well. Although they are the closest homologes of SIZ1 in *Arabidopsis*, they seem to play only a minor role in plant development. Mutants did not differ in growth from wild type and

the majority of data indicate that they do not regulate flowering time. Nevertheless, PIL1 and PIL2 seem to be involved in sulfur metabolism, because data of our cooperation partner Dr. Holger Hesse from the MPI in Golm suggest altered levels of sulfur pathway components in *pil1pil2* double mutants. This preliminary data have yet to be confirmed by sulfur starvation experiments of the *pil1pil2* plants that are conducted at the moment in the laboratory of Holger Hesse.

- The SUMO protease EL1 was investigated. Its localization in the cytoplasm, often in vesicular structures, was shown. The flowering time of different *el1* mutants was also analyzed, but did not differ from wild type neither under a long day nor under a short day regime. Compared to wild type, an *el1* mutant line in the Wassilewskija background showed thinner stems due to an increased ratio of vascular tissue to mesophyll, but this difference in shoot thickness was not observed for *el1* mutants in the ecotype Columbia. It is remarkable that EL1 is the closest homolog of the SUMO protease ESD4 in *Arabidopsis*, but has an entirely different localization and no function in flowering time control, implying a different subset of substrates for these two similar enzymes.

- The growth defect of *esd4* mutants was analyzed. It was shown previously that a similar phenotype of dwarfish growth with reduced apical dominance observable in mutants of the SUMO ligase SIZ1 is due to an increased level of salicylic acid. *Esd4* mutants were therefore crossed with plants defective in salicylic acid biosynthesis. The ensuing *esd4sid2* double mutants did not differ from the *esd4* parent in growth characteristics. This implies that the growth defect of *esd4* plants is independent of salicylic acid signaling. Although ESD4 seems to be the major SUMO protease in *Arabidopsis*, while SIZ is the most important SUMO ligase, both seem to act

in different pathways concerning growth control. To verify these results, an experiment is on-going in which the *esd4* mutants were crossed to plants carrying the bacterial *nahG* gene, coding for salicylic acid hydroxylase, which reduces salicylic acid levels by degrading this phytohormone.

- In cooperation with Prof. Ulla Bonas and Robert Szczesny from the Martin-Luther-University in Halle, Factor X, an effector protein of the bacterial plant pathogen *Xanthomonas campestris* was analyzed for SUMO, Ubiquitin and Rub1 protease activity. During *in vitro* experiments, Factor X was not able to cleave any of the tested fusion proteins. It has therefore to be assumed that this type III effector does not alter proteins linked to these three modifiers the host plant.

The analysis of the PIL1 and PIL2 proteins and the SUMO protease EL1 revealed that SUMO ligases and proteases exist in *Arabidopsis* that have only minor effects on plant development while strong effects of their closest homologs were reported. This implies on the one hand some redundancy in the SUMOylation pathways, because the knockout of these proteins had only weak effects on plant development. On the other hand, the existence and expression (ESTs exists) of the PILs and EL1 could mean that their influence on SUMOylation might be important during certain developmental phases, or in responses to abiotic or biotic factors not monitored during our experimental settings. This hypothesis is strengthened by the preliminary data of our cooperation partner Holger Hesse, which implicate a function of PIL proteins in sulfur metabolism.

The analysis of ESD4 also showed that, although this protein plays a role in flowering time control via regulation of the transcription factor FLC, which is also regulated by the SUMO ligase SIZ1, both enzymes seem to act in different pathways concerning growth control or hormone signaling. *Siz1* mutants have increased levels of salicylic acid leading to reduced growth and apical dominance,

while an alteration of salicylic acid seems not to be the cause of the similar phenotype of *esd4* mutants.

This work demonstrates that SUMOylation plays an important regulatory role in many, yet unidentified processes and that, although the involved enzymes are often highly similar and might be partly redundant, slight differences exist that allow fine regulation of many different developmental processes and responses to the environment. Although sometimes mutants of those enzymes might resemble each other phenotypically, they might be altered in different pathways.

It becomes clear that SUMOylation plays a vital role in the regulation of many processes in *Arabidopsis*, but only little is known about the exact regulation and the interaction of the different enzymes and substrates. Only the identification of *in vivo* substrates of SUMO as already performed by Dr. Ruchika Budhiraja and their further investigation can answer these questions. With the *in vitro* SUMOylation system and the SUMO1 variant SUMO1 Q90A, which were characterized in this work, two valuable tools are available, which might help to accomplish this task.

5 MATERIAL AND METHODS

5.1 Material

5.1.1 Chemicals

Chemicals used in this work were purchased in laboratory quality from the companies Sigma, Roth, Duchefa, Amersham, Merck, Fluka, Gibco, Roche and Invitrogen.

5.1.2 Vectors

In the following list vectors and plasmids used for the generation of DNA constructs are shown.

Table 5.1 : Vectors used in this work

Vector	Origin	Description
pET9a-d	Novagen	The pET-9a-d(+) vectors carry a N-terminal T7-Tag® sequence and *Bam*H I cloning site. These vectors are the precursors to many pET family vectors.
pET19	Novagen	The pET-19b vector carries a N-terminal His tag sequence followed by an enterokinase site and three cloning sites.
pET-42a,b,c	Novagen	The pET-42 series was used for cloning and high-level expression GST fusion proteins.
pETM30	EMBL protein expression and purification unit	The pETM30 is a vector for expression of proteins with a GST moiety and a His tag.
pDEST17	Invitrogen	This vector was used for expression of proteins and determines resistance to ampicillin.
pQE30	Qiagen	pQE30 was used for expression of His tagged proteins.
pGEX-4T-2	GE Healthcare	pGEX vectors allow the production of GST fusion proteins and confer ampicillin resistance.

Vector	Origin	Description
pLysS, E	Novagen	These two vectors were constructed by insertion of the T7 lysozyme gene into the *Bam*H I site of pACYC184. They are no cloning vectors, but are used in lDE3 lysogenic hosts to suppress basal expression from the T7 promoter by producing T7 lysozyme, a natural inhibitor of T7 RNA polymerase. This allows tight regulation of transgene expression.
pLysSRARE	Novagen	pLysSRARE contains the genes encoding T7 lysozyme (lysS), a chloramphenicol resistance gene and tRNA genes corresponding to rare codons in *E. coli*.
pBlueskript II	Stratagene	This vector was used for routine cloning procedures and determines ampicillin resistance.
pER8	Zuo *et al*., 2000	pER8 is a binary T-DNA cloning vector that carries a β-estradiol inducible promoter. Selection markers of this vector are spectinomycin (bacteria) and hygromycin (*Arabidopsis*).
p3	Yin *et al*., 2007	This vector combines the pBIB backbone with the multiple cloning site of pRT103 (Becker, 1990; Topfer *et al*., 1993) and allows expression under the 35S promoter with three enhancers.
pHi	Schloegelhofer and Bachmair, 2002	This vector was used for constitutive expression of transgenes in *Arabidopsis*, because it carries the CaMV35S promoter with two enhancers..

5.1.3 Oligonucleotides

Oligonucleotides that were obtained from Isogen Life Science or Operon and used in this work are listed in Table 5.2.

Table 5.2 : Oligonucleotides used in this work

Oligonucleotide	Sequence	Description
Primers for the generation of amino acid exchanges in TAF7		
TAF7(K328R)f	GTT GTT GAG TCT TTT **AGA** ACT TAT GAT GAT TGT G	These two primers were utilized for site directed mutagenesis of TAF7 to exchange lysine 328.
TAF7(K328R)r	CAA TCA TCA TAA GT**T CT**A AAA GAC TCA ACA AC	
TAF7(K337R)f	GAT TGT GCA TTA GTC **AGA** ACT GCT GAT ATT GGG	Both oligonucleotides were generated to exchange lysine 337 in TAF7.
TAF7(K337R)r	CCC AAT ATC AGC AGT **TCT** GAC TAA TGC ACA ATC	
TAF7(K369R)f	CTA ACT CCT CCA ATG **AGG** GAT GCT CGA AAG AGG	With those primers the lysine 369 in TAF7 was mutated to arginine.
TAF7(K369R)r	CCT CTT TCG AGC ATC **CCT** CAT TGG AGG AGT TAG	
TAF7(K373R)f	ATG AAG GAT GCT CGA **AGA** AGG AGA TTT CGT CGA	To create TAF7(K373R) these two oligonucleotides were used.
TAF7(K373R)r	CTC GAC GAA ATC TCC T**TC T**TC GAG CAT CCT TCA T	
TAF7(K423,424R)f	GCT AGT AAT GCA AGT **AGG AG**A GTA TCT TCT TCT TC	These two primes allowed the exchange of two neighbouring lysine residues in TAF7.
TAF7(K423,424R)r	GAA GAA GAA GAT AC**T CTC CT**A CTT GCA TTA CTA GC	
TAF7(K435R)f	CCZT ACA CCT GTT GAA **AGG** CCT GAA GCT CCT GAG	These oligonucleotides were generated for the mutation of lysine 435 close to the carboxyl terminus of TAF7.
TAF7(K435R)r	CTC AGG AGC TTC AGG **CCT** TTC AAC AGG TGT AGG	
Oligonucleotides utilized to generated plasmid constructs		
SAE1a fill in 1	CAT GCG AGC ATG GAC GGA GAA GAG CCC GGG ATC C	These oligonucleotides were annealed and inserted into a construct during the creation of pET9d-SAE1aSAE2.
SAE1a fill in 2	TCG AGG ATC CCG GGC TCT TCT CCG TCC ATG CTC G	
GST-S-Flag fill in 1	CCA GAA CCA CTA GTT GAA CCA TCC GAG CGT GGA GGA T	These primers were used in the generation of pET42c-GST-S-Flag.
GST-S-Flag fill in 2	GCT GAA AAT GTT CGA AGA TCG TTT	
GST(K217)R fill in 1	GCT GAA AAT GTT CGA AGA TCG TTT	These oligonucleotides were utilized to generate the amino acid exchange in pET42c-GST(K217R)-S-Flag.
GST(K217)R fill in 2	CCA GAA CCA CTA GTT GAA CCA TCC GAG CGT GGA GGA T	

Oligonucleotide	Sequence	Description
Primers used for sequencing of transgenic plants		
ESD4 start dn	CTA ATG GGT GCC GTA GCG ATC ATT C	These primers anneal in the wild type allele of esd4.
ESD4 up 2	TAT CTG CAG AGG GCA ACA GAC TAA GTT	
sid2-1f	GCA GTC CGA AAG ACG ACC TCG AG	With these olignucleotides the sid2 mutation can be detected. They were obtained from Dr. J. Parker (MPIZ).
sid2-1r	CTA TCG AAT GAT TCT AGA AGA AGC	
SALK LBa1	TGG TTC ACG TAG TGG GCC ATC G	This primer anneals in T-DNA insertions and can therefore be used to detect those.

Above the oligonucleotides used in this work are shown in 5´to 3´direction. In case of the primers utilized for site directed mutagenesis, the bases encoding the mutated residue are bold.

5.1.4 Plasmid Constructs

The latter enumerations show plasmids used for the overexpression of recombinant proteins in *E.coli* or for *in planta* protein expression. If not stated otherwise, all expressed proteins carry a Histidine tag.

Table 5.3 : Plasmid constructs utilized in this work

Plasmid construct	Origin	Description
Enzymes and SUMO moieties used for in *vitro* SUMOylation assays		
pQE30-SCE	Dr. A. Bachmair	This plasmid allows the overexpression of untagged SCE.
pQE30-SCE(C94S)	Dr. A. Bachmair Dr. C. Hardtke, University of Lausanne	This construct encodes a mutated SCE(C94S) that is no longer catalytically active and carries no tag.
pET9d-SAE1bSAE2	Dr. A. Bachmair	This plasmid resembles bacterial di-cistronic constructs and allows overexpression of SAE1b and SAE2 in equal amounts.
pET9d-SAE1aSAE2	**This work**	The backbone of this construct is the pET9d-SAE1bSAE2 plasmid generated by Dr. A. Bachmair. To obtain the analogous clone with isoform SAE1a, a *Pvu* II – *Sal* I fragment of the original plasmid was inserted into *Ecl* 136II – *Sal* I digested vector pSK. The resulting construct was digested with *Nco* I and *Sal* I, and oligonucleotides CAT GCG AGC ATG GAC GGA GAA GAG CCC GGG ATC C (SAE1a fill in1) and TCG AGG ATC CCG GGC TCT TCT CCG TCC ATG CTC G (SAE1a fill in 2) were annealed and inserted. After *Sap* I and *Sma* I digest, a *Sap* I – *Pme* I fragment from the SAE1a cDNA was inserted. Finally, a *Bsr* GI – *Bam* HI fragment from this construct was used to replace the *Bsr* GI – *Bam* HI fragment of pETSAE1b2, to result in pETSAE1a2.
pDEST17-SIZ1	Dr. Y. Fu-Fu	pDEST17-SIZ1 encodes a functional fragment of the SUMO ligase SIZ1 that contains the SP-RING domain, but neither the SAP nor the PHD domain.
pET9d-tag3-SUMO1	Dr. A. Bachmair, Dr. R. Budhiraja	This construct allows the expression of SUMO1 moieties carrying a triple HA tag.
pET9d-tag3-SUMO1 Q90A	Dr. A. Bachmair, Dr. R. Budhiraja	This construct is identical to pET9d-tag3-SUMO1 except for an amino acid exchange at postion -4 of the SUMO moiety.
pET9d-tag3-SUMO3	Dr. A. Bachmair, Dr. R. Budhiraja	pET9d-tag3-SUMO3 encodes a SUMO3 fusion protein with a triple HA tag.
pET9d-tag3-SUMO5	Dr. A. Bachmair, Dr. R. Budhiraja	This plasmid is similar to the other pET9d-tag3-SUMO constructs and encodes a SUMO5 fusion protein.

Tag fusions analyzed for *in vitro* SUMOylation		
pET42c-UBC27	Dr. A. Bachmair	With this construct not only the UBC27 protein could be expressed but it was also used to produce the Flag epitope for analysis of *in vitro* SUMOylation.
pET42c	Novagen	The commercially available plasmid was used to generate a GST-S peptide fusion protein.
pET42c-GST-S-Flag	**This work**	To generate a plasmid for the expression of GST-S-Flag protein, a fragment (*Nde* I and *Kpn* I digested) of the vector pET42c was inserted in the vector pET42c-UBC27after a *Nde* I and *Kpn* I digest.
pET42c-GST(K217R)-S-Flag	**This work**	pET42c-GST-S-Flag was changed by insertion of a *Swa* I - *Spe* I PCR-generated fragment (oligos GCT GAA AAT GTT CGA AGA TCG TTT and CCA GAA CCA CTA GTT GAA CCA TCC GAG CGT GGA GGA T)with the respective K to R mutation (AAA to CGC).
pET42c-GST(K217R)-Flag	**This work**	For expression of GST-Flag, vector pET42c-GST(K217R)-S-Flag was digested with *Mfe* I and *Bgl* II, treated with Klenow fragment of *E. coli* DNA polymerase to delete the sequence coding for the S peptide, and re-ligated to give pET42c-GST(K217R)-Flag.
Substrates tested for their *in vitro* modification by SUMO		
pET42c-RRM1	Dr. A. Bachmair	This construct encodes the protein RRM1 (At3g56860) carrying a Flag epitope.
pET42c-RRM2	Dr. A. Bachmair	This plasmid allows recombinant expression of RRM2 (At2g41060) as a GST fusion protein.
pET-LA	Dr. A. Bachmair	With the construct pET-LA, the protein LA (At2g43970) can be expressed as a GST fusion.
pET-TAF	**This work**	At first, an *Nco I – Not I* fragment from pUNI clone U63389 (Yamada *et al*., 2003) was inserted into *Nco* I and *Not* I digested vector pET42c to generate a plasmid encoding for version of TAF carrying the S peptide. Thereafter, the ensuing vector was digested with *Bgl* II and *Mfe* I, treated with Klenow fragment and relegated to delete the S peptide from this construct to retrieve pET42c-TAF
pET-TAF(K328R)	**This work**	This construct, encoding for a mutated variety of TAF7, was obtained by site directed mutagenesis of pET-TAF.
pET-TAF(K337R)	**This work**	This construct, encoding for a mutated variety of TAF7, was obtained by site directed mutagenesis of pET-TAF.
pET-TAF(K369R)	**This work**	This construct, encoding for a mutated variety of TAF7, was obtained by site directed mutagenesis of pET-TAF.
pET-TAF(K373R)	**This work**	This construct, encoding for a mutated variety of TAF7, was obtained by site directed mutagenesis of pET-TAF.

pET-TAF(K423,424R)	**This work**	This construct, encoding for a mutated variety of TAF7, was obtained by site directed mutagenesis of pET-TAF.
pET-TAF(K435R)	**This work**	This construct, encoding for a mutated variety of TAF7, was obtained by site directed mutagenesis of pET-TAF.
pET-TAF(K373,423,424R)	**This work**	To retrieve triple mutants of the TAF7, the pET-TAF(K423,424R) plasmid was utilized for further mutagenesis.
pET-TAF(K423,424,435R)	**This work**	To retrieve triple mutants of the TAF7, the pET-TAF(K423,424R) plasmid was utilized for further mutagenesis.
pET-TAF(K373,423,424,435R)	**This work**	This plasmid, which allows expression of a quadruple mutant of TAF7, was obtained by using an already existing construct, encoding for a triple mutant, as a template for site directed mutagenesis.
pET42c-AML5	Dr. A. Bachmair	The open reading frame of At1g29400 was inserted into pET42c between *Sac* II and *Sac* I sites (sequence CCG CGG GTC AT**A TG** ... till stop codon **TGA**, ending at Sca I site of cDNA).
pET-Rxt3	Dr. A. Bachmair	The open reading frame of At5g08450 was inserted into pET42c between *Mfe* I and *Bam* HI sites (sequence CAA TTG GTC AT**A TG** ... till stop codon, ending with Bam HI of cDNA).
pET42c-NAF	Dr. A. Bachmair	This construct codes for a fusion protein of NAF (At2g19480) and the Flag epitope.
pET42c-IAA	Dr. A. Bachmair M. Lehnen	An *Eco* NI Klenow – *Bsr* GI fragment from the cDNA of At1g51950 was inserted into *Bgl* II Klenow – *Asp* 718 treated vector pET42c.
pET-CO N terminus	Dr. A. Bachmair M. Lehnen	An amino-terminal fragment generated by PCR from the CO open reading frame (CAT **ATG** ...TTC CCT AAT TCA GGT ACC) *Nco* I to *Kpn* I was inserted between *Nco* I and *Kpn* I sites of vector pET42c-GST-S-Flag. The ensuing protein is no GST fusion, but has a FLAG tag.
pET-CO mid	Dr. A. Bachmair M. Lehnen	A PCR-generated *Spe* I *Not* I fragment encompassing the middle part of CO (ACT AGT CAT CAG CGA ... CCC TGC AAG CGC GGC CGC) was inserted into *Spe* I - *Not* I digested vector pET42c.
pET-CO C terminus	Dr. A. Bachmair M. Lehnen	A PCR-generated *Mfe* I *Xho* I fragment encompassing the carboxyl-terminal part of CO (CAA TTG CTC AAC AGA ATT ... CCT TCA TTC TGA CTC GAG) was inserted into *Mfe* I *Xho* I digested vector pET42c.
pET-Hap2a	Dr. M. Horvarth, Dr. C. Koncz	This construct was obtained from the laboratory of Dr. Czaba Koncz and allows the expression of Hap2a (At5g12840) fused to the GST moiety.

pETM30-SVP	Dr. P. Huijser	The plasmid allows expression of SVP, cloned into pETM30 vector to obtain a His tag - GST fusion protein.
pET-FLC pET42c-COP1 frag.	Dr. A. Bachmair	This plasmid allows the expression of FLC (At5g10140) fused to the FLAG epitope.
pET-PFU1	Dr. A. Bachmair, K. Eifler	pET-PFU1 allows expression of the protein PFU1 (At3g15355).
pET-β glucuronidase	Dr. A. Bachmair M. Lehnen	A fragment from At1g75940 cDNA encompassing start ATG to Eco RI after the stop codon (sequence CCG CGG GTC ATA**A TG** ... till stop codon **TGA**, ending at *Eco* RI site of cDNA) was inserted between *Sac* II and *Eco* RI sites of pET42c.
Other plasmid constructs		
p3-GFP-EL1	**This work**	To create this construct a GFP-EL1 fragment (Xho I /Sfo I) was inserted into Xho I/ Sma I digested vector p3. The insert was obtained from the plasmid pENSG-GFP DH (from Dr. Yong Fu-Fu). The resulting plasmid allowed transient expression of a GFP-EL1 fusion protein in *N. benthamiana.*
p3-EL1-GFP	**This work**	The plasmid 35-S-EL1-GFP (Dr. Yong Fu-Fu) was digested with Xho I and Spe I and the resulting fragment was inserted into Xho I/ Sma I digested vector p3. This construct was used for transient expression of EL1-GFP.
pQE130-Myc-Ub-Rub1	Dr. H.-P. Stuible	This plasmid was utilized to express a Rub1 fusion protein to test the protease activity of Factor X.
pET-ESD4	Dr. Y. Fu-Fu	This construct allows the expression of a functional fragment of the SUMO protease ESD4.
pET-FLC-SUMO1	Prof. G. Coupland	This fragment allowed the expression of a SUMO1 fusion protein to test the activity of Factor X.
pET-FLC-SUMO3	Prof. G. Coupland	pET-FLC-SUMO3 was used, to analyze the ability of Factor X to cleave SUMO3 fusions.
pDEST17-Factor X	Prof. U. Bonas R. Szczesny	This construct encodes the potential SUMO protease Factor X.
pDEST17-Factor mut	Prof. U. Bonas R. Szczesny	This construct codes for a variant of Factor X, in which the catalytic residue is mutated.

5.1.5 *Escherichia coli* strains

The *Escherichia coli* strains used in this work are listed in Table 5.4.

Table 5.4 : *E. coli* strains

Strains for plasmid maintenance		
Strain	**Origin**	**Genotype**
XL1 blue	Stratagene	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lac*IqZΔ*M15* Tn*10* (Tetr)]
XL10 Gold	Stratagene	Tetr Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lac*IqZΔ*M15* Tn*10* (Tetr) Tn5 (Kanr) Amy]
DH5α	Invitrogen	F- ϕ80*lac*ZΔM15 Δ(□*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-
Strains for protein expression		
BL21	Stratagene	F– *dcm ompT hsdS*(rB– mB–) *gal*
BL21 (DE3)	Invitrogen Statagene	F– *dcm ompT hsdS*(rB– mB–) *gal* λ(DE3)
BL21 (DE3) pLysS	Invitrogen Stratagene	F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3) pLysS (Cam^R)
Rosetta(DE3) pLysS	Novagen	F^- *ompT* $hsdS_B$(r_{B^-} m_{B^-}) *gal dcm* (DE3) pLysSRARE (Cam^R)
Strains for sexual conjugation		
S17-1	ATTC	*recA pro hsdR RP4-2-Tc::Mu-Km::Tn7* (chromosomally integrated *tra* genes)

5.1.6 *Agrobacterium tumefaciens* strains

For transient expression of EL1-GFP fusion proteins in *Nicotiana benthamiana* the *Agrobacterium tumefaciens* strain C58C1 pCV2260 (obtained from D. Staiger, TU Zurich, Switzerland) was used.

5.1.7 *Arabidopsis thaliana* Ecotypes

In this work, the *Arabidopsis* ecotypes Columbia 0 and Wassilewskija were utilized as background for mutations and as controls in different experiments.

5.1.8 *Arabidopsis thaliana* mutant lines

The *Arabidopsis* mutants which were described in this work are listed below.

Table 5.5: *Arabidopsis thaliana* lines

Mutant	Origin	Description
siz1a	Dr. A. Bachmair SALK_065397 (At5g60410)	This line is mutated in the siz1 gene, and has a rather strong phenotype.
siz1c	Dr. A. Bachmair SALK_034008 (At5g60410)	This line is mutated in the siz1 gene, with an obvious phenotype.
siz1f	Dr. A. Bachmair GABI-Kat line 217A09 (At5g60410)	This line is mutated in the siz1 gene, but confers to a rather mild phenotype in comparison to *siz1a* or *siz1c*.
pil1a	Dr. A. Bachmair GABI-Kat line 339H11	This mutant line originated of a GABI-Kat line and was selected for an insertion within the PIL1 (At1g08910).
pil1b	Dr. A. Bachmair SALK_147797	This line carries a mutation in the pil1 gene.
pil1c	Dr. A. Bachmair SAIL_738_B09	This line carries a mutation in the pil1 gene.
pil2a	Dr. A. Bachmair SALK_043892	This line carries a mutation in the pil2 gene (At5g41580).
pil2b	Dr. A. Bachmair GABI-Kat line 712B09	This line carries a mutation in the pil2 gene (At5g41580).
pil1a pil2b	Dr. A. Bachmair	This line was generated via crossing the respective single mutants.
pil1c pil2a	Dr. A. Bachmair	This line was generated via crossing the respective single mutants.
esd4	Prof. G. Coupland *esd4-2* SALK_032317	This line carries a mutation in the esd4 gene (Reeves *et al*., 2002).
el1a	Dr. Y. Fu-Fu FLAG 201D11 (in Ws background)	The line has an insertion in the first exon of esd4 and is in the genetic background of Wassilewskija.
el1b	Dr. A. Bachmair SAIL_318_C01 N814746 line 1	This mutant line was generated from the homozygous offspring of the SAIL line, which carries the T-DNA insertion in an intron of the coding region.
el1c	Dr. A. Bachmair SAIL_318_C01 N814746 line 2	This mutant line was generated from the homozygous offspring of the SAIL line *el1b*.

If not stated, all mutations are in the genetic background of the ecotype Columbia.

5.1.9 Antibodies

Antibodies utilized in this work are listed below.

Table 5.6 : Antibodies

Antibody	Origin	Description
Primary antibodies		
anti-HA	Roche (11 867 423 001)	This antibody was derived from rat.
anti-GST	SIGMA (G7781)	This antibody is directed against the GST moiety and was produced in rabbit.
anti-Flag	SIGMA (F3165)	That monoclonal anti-Flag antibody was isolated from mouse.
anti-Flag alkaline phosphatase	SIGMA (A9469)	That monoclonal anti-Flag antibody was purified from a murine cell culture.
anti-Myc	US Biological (M9601-31)	This antibody is directed against the human Myc-tag and was generated in mouse.
anti-SUMO1	ABCAM (ab5316)	The polyclonal anti-SUMO1 antibody by ABCAM allows detection of the *Arabidopsis* protein and was generated in rabbit.
anti-SUMO3	ABCAM (ab5317)	The polyclonal anti-SUMO1 antibody by ABCAM allows detection of the *Arabidopsis* protein and was generated in rabbit.
anti-Ubiquitin	Dr. A. Bachmair	This is a polyclonal anti-serum from rabbit directed against *Arabidopsis thaliana* Ubiquitin.
Secondary antibodies		
anti-mouse IgG AP	SIGMA (A3562)	The anti-mouse secondary antibody coupled to alkaline phosphatase was produced in goat.
anti-rabbit IgG AP	SIGMA (A3812)	This antibody was developed in goat using rabbit IgG as immunogen.
-rat IgG AP	Promega (S3731)	This antibody was produced in goat.
anti-rat IgG AP	SIGMA (A8438)	This product was developed in goat.
anti-mouse IgG HRP	SIGMA (A2304)	This antibody is labeled with horse radish peroxidase and was developed in goat.
anti-rabbit IgG HRP	SIGMA (A6154)	This antibody is labeled with horse radish peroxidase and was developed in goat.
anti-rat IgG HRP	SIGMA (A9037)	This antibody is labeled with horse radish peroxidase and was developed in goat.
anti-mouse IR Dye 800	Rockland (610-132-121)	This antibody is coupled to a fluorescent marker and allows signal detection in the 780 nm to 820 nm range. It was developed in goat.

5.2 Methods

5.2.1 Culture and storage of organisms

5.2.1.1 Culture and storage of *Escherichia coli*

Escherichia coli cells were usually grown overnight at 37°C in liquid LB or on LB plates. For selection of transgenic bacteria, the medium was supplemented with the appropriate antibiotics after autoclaving and cooling down to approx. 60°C.
E. coli strains were stored as glycerol stocks. Therefore an overnight culture of bacteria was mixed with an equal amount of 75% glycerol and stored at -80°C.

Antibiotics

Ampicillin	100 $\mu g \cdot ml^{-1}$
Kanamycin	25 $\mu g \cdot ml^{-1}$
Chloramphenicol (in EtOH)	25 $\mu g \cdot ml^{-1}$

LB medium

10 g	bacto-trypton
5 g	yeast extract
10 g	NaCl

The pH is adjusted to 7.0 with NaOH.
H_2O is added to a final volume of 1 l.
For plates 15 $g \cdot l^{-1}$of agar were added

5.2.1.2 TSS competent *Escherichia coli*

To introduce plasmids into *E. coli*, the cells have to be made competent for transformation.

LB medium was inoculated 1:100 from an *E.coli* culture grown overnight. At an OD_{600} of 0.3 to 0.4, cells were harvested by centrifugation (10 min at 1000x g and 4°C). The pellet was resuspended in 1/10 volume of chilled TSS solution and incubated for 5 to 15 min on ice. Aliquots of 100 µl were frozen rapidly in liquid N_2 and stored at -80°C for further use.

TSS solution

10 %	PEG 4000
50 mM	$MgCl_2$
	in 90 ml LB

The pH was adjusted to 6.7 and LB medium was added to a final volume of 95 ml.
After sterile filtration, the TSS solution was stored as 9.5 ml aliquots at -20°C.
Prior to use, 0.5 ml DMSO was added and the medium was stored on ice.

5.2.1.3 **Heat shock transformation of *Escherichia coli***

Heat shock transformation allows the introduction of plasmids into *E. coli*.

An aliquot of TSS competent cells was thawn on ice and incubated with 1 µl plasmid DNA or 15 µl ligation for 10 – 30 min on ice. Afterwards cells were incubated for 2 min at 37°C and put directly on ice. The bacteria were imbibed in 0.75 ml LB medium and incubated for 1 h at 37°C and 750 rpm. An aliquot was plated on LB plates containing antibiotics for selection of transformants.

5.2.1.4 **Culture and storage of *Agrobacterium tumefaciens***

Agrobacteria were incubated at 28°C in YEB medium, containing the appropriate antibiotics. For the *Agrobacterium tumefaciens* C58C1, Rifampicin was always added, because this strain contains a plasmid providing resistance to that antibiotic.

Antibiotics		**YEB medium**	
Kanamycin	25 µg·ml^{-1}	5 g	beef extract
Rifampicin	100 µg·ml^{-1}	1 g	yeast extract
Spectinomycin	50 µg·ml^{-1}	5 g	peptone
Carbicillin	100 µg·ml^{-1}	5 g	sucrose

The pH was adjusted to 7.2 – 7.3 with NaOH. H_2O is added to a final volume of 1 l; 2 – 10 mM $MgSO_4$ (sterile filtrated) was added after autoclavation.

5.2.1.5 **Heat shock competent *Agrobacteria***

Similar to *E. coli* cells, *Agrobacteria* have to be made competent for transformation.

To create heat shock transformation competent *Agrobacteria,* 10 ml YEB (50 µg·ml-1 Rifampicin, 2 mM $MgSO_4$) were inoculated with *A. tumefaciens* and incubated for two days at 28°C. The cells were diluted 1:25 in fresh medium and incubated for about 3.5 hours. Cells were harvested by centrifugation (5000 rpm, 10 min, 4°C) and resuspended in 1 ml precooled YEB (50 µg·ml-1 Rifampicin, 2 mM $MgSO_4$). Aliquots of 200 µl were frozen in liquid N_2 and stored at -80°C.

5.2.1.6 **Transformation of *Agrobacteria***

Plasmid constructs can be introduced into *Agrobacteria* by heat shock treatment.

An aliquot of heat shock competent *Agrobacteria* was thawn at room temperature and then transferred onto ice. After mixing with 2 -5 µg DNA, the cells were frozen in liquid N_2 for 1 min and then directly transferred to 37°C for 5 min. The cells were imbibed in 1 ml YEB medium (50 µg·ml-1 Rifampicin, 2 mM $MgSO_4$) and incubated for 2 h at 28°C, before they were spread on YEB plates containing the antibiotics necessary for selection of transformation.

5.2.1.7 **Sexual transformation of *Agrobacteria***

During sexual transformation of *Agrobacteria*, a plasmid is transferred from *E.coli* into *Agrobacteria* via sexual conjugation.

For both microorganisms a liquid culture was inoculated from a preculture grown overnight and incubated until an OD_{540} of approx. 0.5. Then 0.5 ml of each culture were carefully mixed. Big drops were set on a YEB plate (2 mM $MgSO_4$, 0.04 mM $CaCl_2$) that contained calcium, to promote pili formation. The plate was dried under the sterile bench and incubated for 1 day at 28°C. The cells were resuspended in 5 ml YEB medium and different dilutions were spread on YEB plates, containing the necessary antibiotics for selection of positive transformants and Rifampicin to eliminate the *E. coli* bacteria.

5.2.1.8 **Culture of *Arabidopsis thaliana* plants**

Arabidopsis plants were either grown on 1% ara medium or on soil under long day (16 h light) or short day (8 h light) conditions. For selection of transgenic plants, Hygromycin was added to the medium, while Claforan was used to inhibit growth of *Agrobacteria* after plant transformation.

Antibiotics

Hygromycin	25 μg·ml^{-1}
Claforan	200 μg·ml^{-1}

500x Vitamin mix

5 g	myo-inositol
1 g	thiamine
50 mg	nicotinic acid
10 mg	biotin

H_2O was added to a final volume of 100 ml and after sterile filtration aliquots were stored at -20°C.

1% ara medium

4.3 g	MS salt
10 g	sucrose
0.5 g	MES
8 g	agar for plates

The pH was adjusted to 5.7 with KOH. H_2O was added to a final volume of 1 l. After autoclaving, the medium was supplemented with vitamin mix prior to use.

5.2.1.9 **Seed sterilization**

Small batches of seeds were shaken in 0.5 ml sterilization solution (3-5% calcium hypochlorite and 1/1000 volume of 20% Trition X-100) for 15 min at room temperature. They were washed twice with 0.5 ml sterile H_2O and once with 150 μl H_2O and then dried in a sterile hood. Up to further use sterilized seeds were stored at 4°C.

5.2.1.10 **Floral Dip Transformation of *Arabidopsis thaliana***

This technique allows the introduction of a DNA construct into plants by using *Agrobacteria*.

Nine plants per pot were grown on soil. The first shoots were cut to increase the total number of shoots. After 7-10 days, plants were ready for transformation. For this, 20 ml YEB medium (required antibiotics, 10 mM $MgSO_4$) were inoculated for 2 days at 28°C with *Agrobacteria*, which carried the construct to be introduced into plants. Afterwards 100 ml fresh medium were inoculated with 2 ml of this preculture and incubated overnight. The cells were harvested (5000rpm, 15 min, RT) and resuspended in 200 ml 5% sucrose + 0.05% Silvet. Plants were dipped for 1-2 min in this solution and after drying transferred to the greenhouse.

5.2.1.11 **Crosses of plants**

To cross plants, the parent plants were grown in the greenhouse. For the mother plants, all parts of the flower except the gynoceum were removed before the flower opens. Then the free gynoceum was pollinated by running a stamen of the father plant over it.

5.2.1.12 **Induction of transgene expression by β-estradiol**

To avoid constant expression of harmful constructs, it can be favorable to express transgenes in *Arabidopsis* under control of an inducible promoter.

To induce transgenes under control of a β-estradiol inducible promoter, plants were usually grown on 1% ara plates containing 5 μM β-estradiol. For liquid induction, plants were first grown on plates and then either submerged or transferred into 24 well plates with medium supplemented with β-estradiol. Normally, plants were harvested 1-3 days after induction and analyzed for production of transgenic proteins by Western blot.

5.2.1.13 **Flowering time measurement**

To analyze the flowering time of different mutants, plants were sown in flat pots on soil and vernalized for 2 days. Seedlings were singled in 7x7 cm pots and grown in long day or short day growth chambers. When the first flower opened, the rosette leafs were counted.

5.2.1.14 **Tissue stain**

To detect differences in the tissue compositon of *el1* mutant and wild type shoots, stains with either only Safranin T or a combination of Astralblue and Safranin T were conducted. Shoot sections were cut with a razor blade and stored in water up to further use. The slices were incubated in a solution of 1% Astralblue in 0.5 acidic acid for 20 min at room temperature and then washed with H_2O until the supernatant stayed clear. Afterwards they were transferred into a dye of 1%

Safranin T in 50% EtOH for 10 min. The cuttings were washed with EtOH until the supernatant was no longer tinted. Then, they were washed four times with H_2O and stored in water. If the samples were only stained with Safranin T, only the last steps of the procedure were conducted.

5.2.2 Working with DNA

5.2.2.1 **Plasmid Preparation (from *E. coli*)**

Plasmid constructs were purified from transgenic *E. coli* strains with the kit "Nucleospin Plasmid" (Macherey-Nagel) according to the manufacturer´s manual. The DNA was eluted with 50 µl sterile H_2O and stored at -20°C up to further use.

5.2.2.2 **Small scale DNA isolation from *Agrobacteria tumefaciens***

After transformation *Agrobacteria* were tested for the correct plasmid.

Therefore 5 ml YEB medium (50 µg·ml-1 Rifampicin, 50 µg·ml-1 Kanaymcin or Spectinomycin, 2 mM $MgSO_4$) were inoculated with *Agrobacteria* and incubated at 28°C until an OD_{600} of 0.8 was reached. After cell harvest, DNA was isolated using a DNA Minikit ("Nucleospin Plasmid", Macherey-Nagel; "Plasmid Mini Kit", Peqlab) according to the manual.

5.2.2.3 **Quick DNA purification from *Arabidopsis***

Using this method, DNA can be manually purified from *Arabidopsis*.

A leaf was frozen in liquid N_2 and grinded with sand and 200 µl Quick DNA purification buffer. After centrifugation (14000 rpm, 5 min, RT), the supernatant was transferred to a fresh tube and mixed with 200 µl isopropanol by shaking for 5 min at room temperature. After a second centrifugation step (14000 rpm, 5 min, RT), the pellet was washed with 0.5 ml 70% EtOH. The dried pellet was resuspended in 70 µl H_2O. After boiling for 5 min at 65°C, it was stirred with a

pipet tip and again heated for 5 min. After centrifugation (14000 rpm, 2 min, RT), the supernatant was transferred to a fresh tube.

5.2.2.4 **DNA isolation from Arabidopsis thaliana**

If several DNA samples from *Arabidopsis* were prepared, the DNA was isolated with the BioSprint96 and the according "BioSprint 96 DNA Plant Kit" (Qiagen). The program P_100, during which the sample is eluted in 100 µl H_2O, was used, to get highly concentrated total plant DNA.

5.2.2.5 **Agarose gels**

Agarose gels were used to separate DNA fragments or strands according to their size and to quantify DNA by visualization with UV light after staining with ethidium bromide.

Dependent on the size of the fragments concentrations of 0.8-2% agarose were melted in 1x TAE. After cooling down, ethidium bromide was added to a final concentration of 0.1 $\mu g \cdot ml^{-1}$ and the gel was poured. Prior to loading, the samples were mixed with 1/6 volume of 6x DNA loading buffer.

50x TAE stock

242 g	Tris base
57.1 ml	glacial acetic acid
37.2 g	$Na_2EDTA \times 2\ H_2O$
ad 1 l	H_2O

6x DNA loading buffer

50%	glycerol
0.2 M	EDTA pH 8.0
0.005%	Orange G

5.2.2.6 **Extraction of DNA from agarose gels or purification of PCR products**

The DNA was separated on an agarose gel and the desired fragments were cut out or a certain fragment was amplified via PCR. In both cases the DNA of interest was purified with the kit "Nucleospin Extract II" (Macherey-Nagel) according to the manual. In most cases, the DNA was eluted in 15 µl elution buffer.

5.2.2.7 **DNA Restriction**

DNA was digested to use it in subsequent experiments e.g. ligation or to search for correct transformants.

Restriction analysis of DNA was performed with DNA endonucleases of the companies New England Biolabs and Fermentas. If possible, digestions with more than one enzyme were carried out in parallel. Otherwise double digests were performed stepwise and the DNA was precipitated and resolved in another buffer if necessary.

For partial digest, samples were taken at different time points and monitored by agarose gels.

5.2.2.8 **Precipitation of DNA**

DNA was precipitated to receive higher concentrations or to change buffer conditions. Therefore the DNA was mixed with 1/10 volume 3 M KoAC (pH 4.9 – 5.3) and 2-3 volumes 96% EtOH and incubated at -20°C for at least 30 min, but preferably over night. The DNA was pelleted by centrifugation (14000 rpm, 15 min, 4°C) and washed with 70°C EtOH. Afterwards, the DNA was pelleted again (14000 rpm, 5 min, 4°C) and the supernatant was carefully removed. The dried pellet was resolved in sterile H_2O.

5.2.2.9 **Ligation of DNA fragments**

Ligases facilitate the assembly of e.g. vector backbone and insert.

Usually, ligations were performed with 1-2 U T4 ligase (Roche) at 16°C over night or at least 3 h. Insert and vector backbone were used in a ratio of approx. 3:1.

5.2.2.10 **Polymerase chain reactions (PCR)**

PCR, Polymerase chain reaction, was used to amplify distinct DNA fragments. According to the length of fragments and further use, different DNA polymerases

and PCR programs were used. Primers were obtained from Isogen Life Science or Operon.

PCR with Brown-Taq polymerase

For general PCR reaction, home-made Taq polymerase and buffer, provided by Dr. Ian Searle, was used.

PCR mix for Brown-Taq

4 µl	Template (genomic DNA)
5 µl	Brown-Taq buffer
5 µl	2.5 mM dNTPs
1 µl	100 pmol·µl^{-1} primer 1
1 µl	100 pmol·µl^{-1} primer 2
0.5 µl	Brown-Taq
ad 50 µl	H_2O

10x Brown-Taq buffer

500 mM	KCl
100 mM	Tris-HCl pH 8.3
0.1%	gelatin
0.5%	Tween 20
250 µg·ml-1	BSA

After sterile filtration aliquots were stored at -20°C.

PCR with commercially available Taq polymerase

In some reactions, LA-Taq polymerase provided by TaKaRA Bio Inc., Japan, or GoTaq polymerase (Promega) were used with the supplied buffers.

PCR mix for LA-Taq

4 µl	template
5 µl	LA-Taq buffer
5 µl	25 mM $MgCl_2$
8 µl	2.5 mM dNTPs
1 µl	100 pmol·µl-1 primer 1
1 µl	100 pmol·µl-1 primer 2
0.5 µl	LA-Taq
ad 50 µl	H_2O

PCR mix for GoTaq

4 µl	template
5 µl	GoTaq buffer green
4 µl	2.5 mM dNTPs
0.5 µl	100 pmol·µl-1 primer 1
0.5 µl	100 pmol·µl-1 primer 2
0.25 µl	GoTaq
ad 50 µl	H_2O

Colony-PCR

Colony-PCR is a quick method to test many different clones after transformation.

As a template a colony was picked with a pipette tip and dissolved in 10 µl H_2O. The following PCR reaction was performed using BrownTaq polymerase and an elongated denaturing step of 10 min in the beginning.

PCR programs

PCR programs were chosen according to the length of the fragment and the used DNA polymerase.

Short programm

94°C	4 min	
94°C	30 s	x 34 repeats
54°C	30 s	
72°C	1 min	
72°C	10 min	
8°C	for ever	

Long programm

95°C	5 min	
95°C	30 s	x 31 repeats
55°C	30 s	
68°C	8 min	
68°C	10 min	
8°C	for ever	

5.2.2.11 **Site directed mutagenesis**

Site directed mutagenesis is a valuable tool to introduce base pair exchanges into plasmids via PCR with misannealing primers to obtain proteins with single amino acid exchanges later on.

To perform site directed mutagenesis the kit "Quik Change II XL Site directed Mutagenesis" (Stratagene) was used according to the manual.

PCR mix for site directed mutagenesis

5 µl	10 ng·µl-1 template
5 µl	buffer
3 µl	Q solution
1 µl	dNTPs
4 µl	10 µM primer 1
4 µl	10 µM primer 2
1 µl	polymerase
ad 50 µl	H_2O

Mutagenesis Program

95°C	1 min	
95°C	50 s	x 18 repeats
60°C	50 s	
68°C	20 min	
68°C	7 min	
6°C	for ever	

5.2.2.12 **Sequencing**

DNA was sequenced by the "Automatic DNA Isolation and Sequencing (ADIS)" service unit of the MPIZ, Cologne.

DNA sequences were determined on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377, 3100 and 3730 sequencers using BigDye-terminator v3.1 chemistry. Premixed reagents were from Applied Biosystems. Oligonucleotides were purchased from Isogen Life Science or Operon.

5.2.2.13 **Detection of the *sid2* mutation in *Arabidopsis***

To test, whether plants carried the *sid2* mutation, a PCR was performed with the sid2-1 primers using a PCR program with 40 cycles. The resulting fragment was digested with *Mun* I to detect the loss of a restriction site in the mutants. This method and the *sid2* mutants were kindly given to our group by Dr. Jane Parker.

5.2.3 Working with proteins

5.2.3.1 **Expression of recombinant proteins in *Escherichia coli***

For further experiments with recombinant proteins those were produced in transgenic *E. coli* cells.

LB medium supplemented with the necessary antibiotics was inoculated with a preculture grown overnight and incubated at 37°C or room temperature up to an OD_{600} of 0.6 – 0.8. After an aliquot was taken for later comparison, production of proteins was induced by adding IPTG to a final concentration of 1 mM. The cells were harvested after 3 h and stored at -20°C or -80°C prior to further use.

5.2.3.2 **Protein purification of recombinant proteins via His tag**

His tag-fusion proteins were preferably purified via Ni-NTA agarose (Qiagen), because this is a rather quick and cheap method.

In general, the purification was performed as suggested by the manufacturer´s manual, but a final concentration of 1 mM PMSF was added to the lysis buffer and 0.5 mM PMSF to the wash buffer. In case of recombinant SUMO activating enzyme all buffers were additionally supplemented with 1 mM ATP to increase the interaction of the two enzyme subunits.

5.2.3.3 **Protein purification of recombinant proteins via FLAG tag**

This method was used to purify potential substrates carrying a FLAG tag from *in vitro* SUMOylation reactions prior to mass spectrometric analysis.

For protein purification via FLAG tag, the Anti-Flag M2 Affinity Gel provided by SIGMA was used. First, the resin was treated twice with 1x TBS. Binding was performed either for 2 h or overnight at 4°C in batch with FLAG tag binding buffer. After the resin was washed three times with TBS, the proteins were eluted by boiling 3 min at 98°C in protein loading sample buffer.

The amount of resin was dependent on the amount of protein solution, e.g. for 100 µl of protein sample 20 µl of resin were used.

FLAG tag binding buffer

50 mM	Tris-HCl pH 7.5
150 mM	NaCl
1 mM	EDTA
1%	Triton X-100

1x TBS pH 7.5

50 mM	Tris-HCl pH 7.5
150 mM	NaCl

5.2.3.4 **Purification of recombinant SUMO conjugating enzyme**

The SUMO conjugating enzyme (SCE) was produced in *E.coli* without any tag, because it was impossible to express this enzyme as a stably active fusion protein. The recombinant SCE was produced in a 2 l culture. After harvesting the cells, the pellet was resuspended in 25 ml 50 M Na phosphate buffer pH 6.5/50 mM NaCl and stored at -80°C for at least 1 d. As the SCE is a highly soluble protein, this procedure was sufficient to let the protein leak out of the cells. After thawing, the

suspension was centrifuged at 29000rpm for 1 h at 8°C and the supernatant was transferred to a fresh tube.

First, the SCE was purified via ion exchange. Therefore an SP-Sepharose column was prepared by washing 10 ml SP-Sepharose Fast Flow (Sigma) with 0.5 M Na phosphate buffer pH 6.5, two times with 50 M Na phosphate buffer pH 6.5/50 mM NaCl and finally with 50 M Na phosphate buffer pH 6.5/50 mM NaCl supplemented with 1 mM DTT and Proteinase Inhibitor (1/4 tablette of Proteinase Inhibitor Cocktail, Roche, per 500 ml). The equilibrated matrix was transferred into plastic column (Biorad). The column was loaded with the lysate by gravity flow and later on washed, using a Minipuls 3 pump (Gibson) at a speed that corresponds to a flow of approx. 1.2 ml/min. The system was washed with approx. 100 ml M Na phosphate buffer pH 6.5/50 mM NaCl supplemented with 1 mM DTT and Proteinase Inhibitor, before proteins were eluted with high salt buffer (50 M Na phosphate buffer pH 6.5/300 mM NaCl, 1 mM DTT and Proteinase Inhibitor). Fractions were collected and analyzed via polyacrylamide gelelectrophoresis and subsequent Coomassie stain.

In a second purification step fractions containing the SCE were first pooled and concentrated with a dialysis column (Vivaspin 15 R, 5000 MWCO HY, Sartorius group). The concentrated solution was further purified with the Äkta FPLC System (Amersham Biosciences) and injected onto a S-200 column. Proteins were eluted with FPLC buffer and those containing SCE were concentrated. This purification step was not always performed because it proofed to increase the purity not much.

5.2.3.5 **Storage of purified proteins**

To store proteins for a longer period of time and to conserve their enzymatic activity, a final glycerol concentration of 15% was added and aliquots, small enough to avoid freeze-thaw cycles were frozen in liquid N_2 and stored at -80°C.

5.2.3.6 **Dialysis of proteins**

Proteins were dialyzed to change their buffer and to increase their concentration if necessary.

For protein dialysation of small sample amounts, Vivaspin Columns (Sartorius) were used. Bigger volumes were dialyzed using the dialysis cassettes (Slide-A-Lyzer Dialysis Cassette, Extra Strength, 10000 MWCO, 0.5-3 ml capacity, Pierce).

The buffer volume exceeded the sample volume at least five times.

5.2.3.7 **SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-Polyacrylamide gels were used to separate proteins according to size under denaturing conditions.

Prior to gel loading, the proteins were denatured by heating (5 min, 98°C) in SDS-containing protein loading sample buffer to negatively charge them. Proteins were separated on 10% to 15% polyacrylamide gels using the Biorad "Mini Protean 3" system. Gels were run at 100 V in 1x electrophoresis buffer.

Stacking gel

330 µl	30% acrylamide mix 29:1 (SERVA)
250 µl	1.5 M Tris-HCl pH 6.8
20 µl	10% SDS
20 µl	10% ammoniumpersulfate
2 µl	TEMED
ad 2 ml	H_2O

2x Protein loading sample buffer

50%	glycerine
20 mM	DTT
2%	SDS
125 mM	Tris-HCl pH 6.8
0.003%	bromphenolblue

Separating gel

10%	12%	15%	
1,7 ml	2 ml	2.5 ml	30% acrylamide mix 29:1 (SERVA)
1,3 ml	1,3 ml	1,3 ml	1.5 M Tris-HCl pH 8.8
50 µl	50 µl	50 µl	10% SDS
50 µl	50 µl	50 µl	10% ammoniumpersulfate
4 µl	4 µl	4 µl	TEMED
		ad 5 ml	H_2O

5x Electrophoresis buffer

7.55 g	Tris
36 g	glycine
.5 g	SDS

5.2.3.8 **Bis-Tris protein gels**

For better resolution, 4-12% NuPAGE Novex Bis-Tris Mini Gels from Invitrogen were used according to the manual with the provided buffers. Gels were run at 200 V.

5.2.3.9 **Coomassie stain**

To visualize proteins in gels, the gels were stained with commercially available Coomassie stain (Rotiblue, Roth; Imperial Protein Stain, Pierce; PageBlue Protein Staining Solution, Fermentas) as recommended by the manufacturers.

5.2.3.10 **Western blot**

For detection of proteins by antibodies, Western blots were performed.

The conditions during these experiments were chosen according to the used antibodies. In general, the proteins were separated on a protein gel and then transferred to a membrane by blotting for 1 h with 50 V at 4°C. To block the membrane afterwards, it was incubated for 1.5 h at room temperature or overnight at 4°C in blocking buffer. Incubation with the first antibody was usually performed overnight at 4°C. After three wash steps, the membrane was incubated with secondary antibody for usually 2 h. Before protein detection, it was washed again three times. The antibodies were dissolved in the solution used for the blocking step, while the wash steps were performed with a buffer not supplemented with proteins.

Western blot with alkaline phosphatase-coupled secondary antibodies

Alkaline phoshatase-coupled antibodies process their substrate BCIP to a stable colored product, which allows the detection of signals with the naked eye, but this stain is not quantitative.

If this type of antibodies was used, the proteins were usually transferred onto a PVDF membrane (Immobilon-P, pore size 0.45 µm, Millipore). The membrane was blocked by either 20% new born calf serum/ANT or by 5% or 10% milk powder/PBS. The wash steps were then performed with 1x ANT or 1x PBS, respectively. If necessary, the buffers were supplemented with 0.002% natrium azide to inhibit bacterial growth or with 0.05% Tween 20 for more stringent conditions. The membrane was stained with 10 ml TE buffer pH 8.0 containing 100 µl 110 mM NBT (in 70% DMF) and 120 µl 90 mM BCIP (in DMF) in the dark.

Western blot with horse radish peroxidase-coupled secondary antibodies

Horse radish peroxidase-coupled antibodies can process their substrates to chemiluminescent products that can blacken X-ray films. This method is semi-quantitative depending on the exposure time.

For Western blots with horse radish peroxidase-coupled secondary antibodies, nitrocellulose membrane (Protran BA85, pore size 0.45 µm, Schleicher&Schuell) was used. The membrane was blocked with 5% or 10% milk powder in 1x PBS, ANT or TBS. Signals were detected with the "SuperSignal West Pico Chemiluminescent Substrate" provided by PIERCE on KODAK Biomax XAR films. Alternatively, the signals were detected with the LumiImager system (BioRAD) that allows digital measurement of the signals.

Western blot with IR-Dye-coupled secondary antibodies

The use of IR-Dye-coupled antibodies allows quantitative detection of protein bands.

This technique was performed with the "Odyssey Infrared Imaging System" (Licor Biosciences) according to the manual with the provided blotting buffer and nitrocellulose membrane. Signals were detected by the "Odyssey Infrared Imager" (Licor Biosciences). Applying this technique, it was possible to quantify the cleavage of SUMOylated products by the SUMO protease ESD4. Therefore different samples were separated via Western blot and the substrate protein was detected with IR-Dye-coupled antibody. As a reference, three background signals of the membrane were measured and the ratio of modified to unmodified substrate was calculated, setting the amount of SUMOylated sample in the negative control to 1.0 (or 100%).

1x ANT

150 mM	NaCl
50 mM	Tris-HCl pH 8.0

1x TE pH 8.0

10 ml	Tris-HCl pH 8.0
2 ml	0.5 mM Na_2EDTA pH 8.0
ad 1l	H_2O

1x TBS

50 mM	glycine
150 mM	Tris

10x PBS

80 g	NaCl
2 g	KCl
26.8 g	Na_2HPO_4 x $7H_2O$
2.4 g	KH_2PO_4
ad 1l	H_2O

adjust pH to 7.4 with HCl

Blotting buffer

190 mM	glycine
25 mM	Tris
20%	methanol
0.05%	SDS

5.2.3.11 *In vitro* SUMOylation assays

In vitro SUMOylation assays are a valuable tool to test the modification of potential substrates by SUMO quickly and easily.

In a typical *in vitro* SUMOylation reaction, 100 µg recombinant SUMO, 4 µg SAE, 0.6 µg SCE and 0.15 µg SIZ1 fragment were incubated in an ATP containing buffer at 30°C for 4 h or overnight. 1–10 µg substrate protein, either carrying a FLAG or a GST tag, were added to the reaction. Aliquots of 10 µl were separated by SDS-

PAGE and analyzed by Western blot using antibodies specific for the substrate´s tag.

SUMOylation buffer

20 mM	Tris-HCl pH 7.5
5 mM	$MgCl_2$
5 mM	ATP

5.2.3.12 **Mass spectrometry analysis**

To identify the lysine residue, to which SUMO was covalently attached, SUMOylated proteins were purified and analyzed by Dr. Jürgen Schmidt, Dr. Thomas Colby and Anne Harzen of the mass spectrometry facility of the MPIZ, Cologne. Prior to mass spectrometry analysis, the samples were digested with trypsin.

In-gel tryptic digest

For the tryptic digest, samples were separated by SDS-PAGE. Coomassie-stained bands were excised and treated as described by Shevchenko *et al*., except that the tryptic digest was performed without adding $CaCl_2$. Peptides were extracted with 100 µl 1% trifluoroacectic acid for 30 min at 37°C. The extraction was repeated once with 100 µl 0.1% trifluoroacectic acid/ acetonitrile (1:2). The supernatants were combined and the volume was reduced to 5 µl in a vacuum centrifuge and 20 µl 0.1% trifluoroacectic were added.

LC-MS/MS analysis

Liquid chromatography (LC)-MS data were acquired on a quadrupole-TOF mass spectrometer (Q-Tof II, Micromass, Manchester, United Kingdom) equipped with a Z spray source. Samples were introduced by an Ultimate nano-LC system (LC Packings) equipped with the Famos autosampler and a Switchos column switching module. The column setup comprises a 0.3 mm x 1 mm trapping column and a 0.075 mm x 150 mm analytical column, both packed with 3 µm

Atlantis dC18 (Waters). A sample volume of 10 µl was injected onto the trap column and desalted for 1 min with 0.1% trifluoroacetic acid at a flow rate of 10 µl/min. Peptides were eluted onto the analytical column by a gradient of 2% acetonitrile in 0.1% formic acid to 40% acetonitrile in 0.1% formic acid over 55 min at a flow rate of ca. 200 nl/min, resulting from a 1:1000 split of the 200 µl/min flow. The electrospray ionization interface comprised an uncoated 10 µm i.d. PicoTip spray emitter (New Objective) linked to the HPLC flow path using a 7 µl dead volume stainless mounted onto the PicoTip holder assembly (New Objective). Stable nanospray was established by the application of 1.7 to 2.4 kV to the stainless steel union. The data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Masslynx 4.0. Survey scans of 1 s covered the range from *m*/*z* 360 to 1,200. Doubly and triply charged ions rising above a given threshold were selected for MS/MS experiments. In MS/MS mode the mass range from m/z 50 to 1,200 was scanned in 1 s, and 3 scans were added up for each experiment. Micromass-formatted peak-lists were generated from the raw data by using the Proteinlynx software module. Proteins were identified by searching the NCBI nr public database (National Center for Biotechnology Information) using a local installation of MASCOT 1.9. (Matrix Science). A mass deviation of 0.5 Da was allowed for peptide and fragment ions.

5.2.3.13 **Localization studies of GFP fusion proteins**

Localization of GFP fusion proteins was determined by the local Central Microscopy (CeMic) facility of the MPIZ, Cologne, with the help of Dr. Elmon Schmelzer and Rainer Franzen.

Nicotiana benthamiana leaves were transformed with *Agrobacterium tumefaciens* C58C1 pCV2260 carrying the p3-El1-GFP or the p3-GFP-El1 plasmid, respectively. Images were taken in the 505-520 nm emission range after exiting with an Argon laser (488 nm) and under transmissible light.

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7 ABBREVIATIONS

Commonly used abbreviations derived from the SI or IUPAC nomenclature as well as the single letter code for amino acids are not explained separately. The other abbreviations are listed below.

Table 7.1: Abbreviations used in this work

A. thaliana	*Arabidopsis thaliana*	NDSM	negatively charged amino acid-dependent SUMOylation motif
A. tumefaciens	*Agrobacterium tumefaciens*	PCNA	Proliferating Cell Nuclear Antigen
AP	alkaline phosphatase	PDSM	phosphorylation-dependent SUMOylation motif
CO	CONSTANS	PIL	PIAS-Like
Col	Columbia	PML	promyelocytic leukemia protein
E. coli	*Escherichia coli*	*S. cerevisae*	Saccharomyces cerevisae
EL1	Early in Short Days 4 – Like 1 (homolog of ESD4)	SAE	SUMO activating enzyme
ESD4	Early in Short Days 4 (a SUMO protease)	SCE	SUMO conjugating enzyme
FLC	Flowering Locus C	SD	short day (8 h light per day)
FLD	Flowering Locus D	SIM	SUMO interacting motif
HDAC	histone deacetylases	StUbls	SUMO-targeted Ubiquitin ligases
HRP	horse radish peroxidase	SUMO	Small Ubiquitin related modifier protein
Hs	*Homo sapiens*	Ubl	Ubiquitin-like protein
LD	long day (16 h light per day)	WS	Wassilewskija
NAF	nucleosome assembly factor	WT	wild type

Table 7.2: Amino acid code

A	alanine	Ala	M	methionine	Met
C	cysteine	Cys	N	asparagines	Asn
D	aspartic acid	Asp	P	proline	Pro
E	glutamic acid	Glu	Q	glutamine	Gln
F	phenylalanine	Phe	R	arginine	Arg
G	glycine	Gly	S	serine	Ser
H	histidine	His	T	threonine	Thr
I	isoleucine	Ile	V	valine	Val
K	lysine	Lys	W	tryptophan	Trp
L	leucine	Leu	Y	tyrosine	Tyr

8 SUPPLEMENTARY

8.1 Sequences and SUMOplot results of different proteins

Below the protein sequences and the results of the SUMOplots of tested potential SUMO substrates are shown. Potential SIMs are indicated as well in Table 8.1.

Table 8.1: SUMOplot results of various proteins

SIMs are shown in bold.

gene: At2g41060 **protein: RRM2**

```
  1 MTKKRKLESE SNETSEPTEK QQQQCEKEDP EIRNVDNQRD DDEQVVEQDT
 51 LKEMHEEEAK GEDNIEAETS SGSGNQGNED DDEEEPIEDL LEPFSKDQ**LL**
101 **ILL**KEAAERH RDVANR**IRIV** ADEDLVHRKI FVHGLGWDTK ADSLIDAFKQ
151 YGEIEDCKCV VDKVSGQSKG YGFILFKSRS GARNALKQPQ KKIGTRMTAC
201 QLASIGPVQG NPVVAPAQHF NPENVQRKIY VSNVSADIDP QKLLEFFSRF
251 GEIEEGPLGL DKATGRPKGF ALFVYRSLES AKKALEEPHK TFEGHVLHCH
301 KANDGPKQVK QHQHNHNSHN QNSRYQRNDN NGYGAPGGHG HFIAGNNQAV
351 QAFNPAIGQA LTALLASQGA GLGLNQAFGQ ALLGTLGTAS PGAVGGMPSG
401 YGTQANISPG VYPGYGAQAG YQGGYQTQQP GQGGAGRGQH GAGYGGPYMG
451 R
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K187	GARNA **LKQP** QKKIG	0.80
2	K60	MHEEE **AKGE** DNIEA	0.79
3	K27	QQQQC **EKED** PEIRN	0.50
4	K6	MTKK **RKLE** SESNE	0.44
5	K192	LKQPQ **KKIG** TRMTA	0.31
6	K268	KATGR **PKGF** ALFVY	0.26
7	K290	ALEEP **HKTF** EGHVL	0.17
8	K129	EDLVH **RKIF** VHGLG	0.09

gene: At3g56860 **protein: RRM1**

```
  1 MTKKRKLEGE ESNEAEEPSQ KLKQTPEEEQ QLVIKNQDNQ GDVEEVEYEE
 51 VEEEQEEEVE DDDDEDDGDE NEDQTDGNRI EAAATSGSGN QEDDDDEPIQ
101 DLLEPFSKEQ **VLSLL**KEAAE KHVDVANRIR EVADEDPVHR KIFVHGLGWD
151 TKTETLIEAF KQYGEIEDCK AVFDKISGKS KGYGFILYKS RSGARNALKQ
201 PQKKIGSRMT ACQLASKGPV FGGAPIAAAA VSAPAQHSNS EHTQKKIYVS
251 NVGAELDPQK LLMFFSKFGE IEEGPLGLDK YTGRPKGFCL FVYKSSESAK
301 RALEEPHKTF EGHILHCQKA IDGPKPGKQQ QHHHNPHAYN NPRYQRNDNN
351 GYGPPGGHGH LMAGNPAGMG GPTAQVINPA IGQALTALLA SQGAGLAFNP
401 AIGQALLGSL GTAAGVNPGN GVGMPTGYGT QAMAPGTMPG YGTQPGLQGG
451 YQTPQPGQGG TSRGQHGVGP YGTPYMGH
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K199	GARNA **LKQP** QKKIG	0.80
2	K6	MTKK **RKLE** GEESN	0.44
3	K325	KAIDG **PKPG** KQQQH	0.43
4	K204	LKQPQ **KKIG** SRMTA	0.31
5	K286	KYTGR **PKGF** CLFVY	0.26
6	K308	ALEEP **HKTF** EGHIL	0.17
7	K141	EDPVH **RKIF** VHGLG	0.09

gene: At2g43970 **protein: LA**

```
  1 MADQQTLDSS TPPPTQSDDL SHSHSTSSTT SASSSSDPSL LRSLSLSRLN
 51 AGAPEFVPGR TTPPLPQPPR MIIPPPPPHG MLHMYHHQPP FNTPVLGPVP
101 IQPHLVPVQN HHPHHRFHQH HHHNRHQNQQ YVPVRNHGEY QQRGGVGGEQ
151 EPDLVSKNKD RRDHSKRESK NDQVTETGAS VSIDSKTGLP EDSIQKIVNQ
201 VEYYFSDLNL ATTDHLMRFI CKDPEGYVPI HVVASFKKIK AVINNNSQLA
251 AVLQNSAKLF VSEDGKKVRR ISPITESAIE ELQSRIIVAE NLPEDHCYQN
301 LMKIFSTVGS VKNIRTCQPQ NNGSGAPPAA RSAAKSDGTL FSNKVHAFVE
351 YEIVELAERA VTELSEAGNW RSGLKVRLML KHQTKEPKQG QGRGRKGHDA
401 DVEHEEDDAT TSEQQPIEKQ SDDCSGEWDT HMQEQPIGED QGNEKAAGQR
451 KGRNRGRGKG RGRGQPHQNQ NQNNNHSHNQ NHNHNGRGNH HHHHHHQVGT
501 QPSNNPMNNM EQPGMGKQQP PGPRMPDGTR GFSMGRGKPV MVQAE
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K335	AARSA **AKSD** GTLFS	0.79
2	K158	PDLVS **KKND** RRDHS	0.48
3	K303	CYQNL **MKIF** STVGS	0.45
4	K258	VLQNS **AKLF** VSEDG	0.44
5	K388	HQTKE **PKQG** QGRGR	0.43

gene: At1g55300 **protein: TAF7**

```
  1 MEEQFILRVP PSVSERIDRL LSEDASTSDE IPLDLFFSED GRNGTFMIGN
 51 DEFPASLLDL PAVVESFKTY DDCALVKTAD IGQMIMVREP GDPAPNTVEY
101 RHGLTPPMKD ARKRRFRREP DLNPELVQRV ERDLLNILSG GTVENVSFSF
151 YFRHMMCLYL LLGFSNFIYL NFFWCRYMSK RNQLRTRMLV MQVRKYLLLH
201 LHLLKSLKLL RQALVIQQEL NRREVNQKIL MIQCEFIII
```

gene: At1g29400 **protein: AML5**

```
  1 MDIPHEAEAG AWGILPGFGR HHHPSSDATL FSSSLPVFPR GKLQLSDNRD
 51 GFSLIDDTAV SRTNKFNESA DDFESHSIGN LLPDEEDLLT GMMDDLDLGE
101 LPDADDYDLF GSGGGMELDA DFRDNLSMSG PPRLSLSSLG GNAIPQFNIP
151 NGAGTVAGEH PYGEHPSRTL FVRNINSNVE DSELTALFEQ YGDIRTLYTT
201 CKHRGFVMIS YYDIRSARMA MRSLQNKPLR RRKLDIHFSI PKDNPSEKDM
251 NQGTLVVFNL DPSISNDDLH GIFGAHGEIK EIRETPHKRH HKFVEFYDVR
301 GAEAALKALN RCEIAGKRIK VEPSRPGGAR RSLMLQLNQD LENDDLHYLP
351 MIGSPMANSP PMQGNWPLNS PVEGSPLQSV LSRSPVFGLS PTRNGHLSGL
401 ASALNSQGPS SKLAPIGRGQ IGSNGFQQSS HLFQEPKMDN KYTGNLSPSG
451 PLISNGGGIE TLSGSEFLWG SPNARSEPSS SSVWSTSSTG NPLFSTRVDR
501 SVPFPHQHQN QSRSHHHFHV GSAPSGVPLE KHFGFVPESS KDALFMNTVG
551 LQGMSGMGLN GGSFSSKMAN NGIINSGSMA ENGFSSYRMM SSPRFSPMFL
601 SSGLNPGRFA SGFDSLYENG RPRRVENNSN QVESRKQFQL DLEKILNGED
651 SRTTLMIKNI PNKYTSKMLL AAIDEKNQGT YNFLYLPIDF KNKCNVGYAF
701 INMLNPELII PFYEAFNGKK WEKFNSEKVA SLAYARIQGK SALIAHFQNS
751 SLMNEDMRCR PIIFDTPNNP ESVEQVVDEE SKNMDLLDSQ LSDDDGRERS
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K320	IAGKR **IKVE** PSRPG	0.94
2	K437	HLFQE **PKMD** NKYTG	0.61
3	K720	EAFNG **KKWE** KFNSE	0.48
4	K233	KPLRR **RKLD** IHFSI	0.44
5	K531	SGVPL **EKHF** GFVPE	0.15
6	K636	NQVES **RKQF** QLDLE	0.09

gene: At5g08450 **protein: Rxt3**

```
  1 MSGVPKRSHE EGVTHPSSSS SVAKYPHEDS GSYPKSPHQP VTPPPAQVHH
 51 NHQQPHQHPQ SQSQSQPQPH LQALPHPHSH SHSHSPLAAA ASASAPYEVE
101 SRTVVKVARS EPRDGERRSP LPLVYRSPSL PTTVSSSDPH LTHAPVPMEP
151 RDGAKDGREI RVESRENRSD GREIYGETKR EIQGPKGDRD VKFERSVDDF
201 SGKGNTGSYT RNDGREMYGE TKREIQGPKS DRDAKFERPG DDFSGKSNAG
251 SYTRDTKFDR ENQNYNEQKG EIKMEKEGHA HLAWKEQKDY HRGKRVAEGS
301 TANVDPWVVS RGNPQGPTEV GPKDLSAPVE GSHLEGRETV GENKVDAKNE
351 DRFKEKDKKR KELKHREWGD RDKDRNDRRV SVLVGSVMSE PKEIGREERE
401 SDRWERERME QKDRERNKEK DKDHIKREPR TGAEKEISQN EKELGEASAK
451 PSEQEYVAPE QKKQNEPDNC EKDERETKEK RRERDGDSEA ERAEKRSRIS
501 EKESEDGCLE GEGATEREKD AFNYGVQQRK RALRPRGSPQ TTNRDNVRSR
551 SQDNEGVQGK SEVSIVVYKV GECMQELIKL WKEYDLSHPD KSGDFANNGP
601 TLEVRIPAEH VTATNRQVRG GQLWGTDIYT DDSDLVAVLM HTGYCRPTAS
651 PPPPTMQELR TTIRVLPSQD YYTSKLRNNV RSRAWGAGIG CSYRVERCYI
701 LKKGGGTIEL EPSLTHSSTV EPTLAPMAVE RSMTTRAAAS NALRQQRFVR
751 EVTIQYNLCN EPWIKYSISI VADKGLKKPL FTSARLKKGE VLYLETHSCR
801 YELCFAGEKT IKAIQASQQQ SSHEAMETDN NNNKSQNHLT NGDKTDSDNS
851 LIDVFRWSRC KKPLPQKLMR SIGFPLPADH IEVLEENLDW EDVQWSQTGV
901 WIAGKEYTLA RVHFLSPN
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K273	EQKGE **IKME** KEGHA	0.94
2	K426	KDKDH **IKRE** PRTGA	0.94
3	K192	KGDRD **VKFE** RSVDD	0.93
4	K777	VADKG **LKKP** LFTSA	0.80
5	K348	ENKVD **AKNE** DRFKE	0.79
6	K235	KSDRD **AKFE** RPGDD	0.79
7	K787	FTSAR **LKKG** EVLYL	0.73
8	K702	ERCYI **LKKG** GGTIE	0.73
9	K24	SSSSV **AKYP** HEDSG	0.69
10	K560	NEGVQ **GKSE** VSIVV	0.67
11	K155	EPRDG **AKDG** REIRV	0.62
12	K229	REIQG **PKSD** RDAKF	0.61
13	K186	REIQG **PKGD** RDVKF	0.61
14	K269	QNYNE **QKGE** IKMEK	0.50
15	K35	DSGSY **PKSP** HQPVT	0.50
16	K472	EPDNC **EKDE** RETKE	0.50
17	K844	HLTNG **DKTD** SDNSL	0.50
18	K788	TSARL **KKGE** VLYLE	0.48
19	K591	DLSHP **DKSG** DFANN	0.33
20	K276	GEIKM **EKEG** HAHLA	0.33
21	K703	RCYIL **KKGG** GTIEL	0.31

gene: At2g19480 **protein: NAF**

```
  1 MSNDKDSMNM SDLSTALNEE DRAGLVNALK NKLQNLAGQH SDVLENLTPP
 51 VRKRVEFLRE IQNQYDEMEA KFFEERAALE AKYQKLYQPL YTKRYEIVNG
101 VVEVEGAAEE VKSEQGEDKS AEEKGVPDFW LIALKNNEIT AEEITERDEG
151 ALKYLKDIKW SRVEEPKGFK LEFFFDQNPY FKNTVLTKTY HMIDEDEPIL
201 EKALGTEIEW YPGKCLTQKI LKKKPKKGSK NTKPITKTED CESFFNFFSP
251 PQVPDDDEDL DDDMADELQG QMEHDYDIGS TIKEKIISHA VSWFTGEAVE
301 ADDLDIEDDD DEIDEDDDEE DEEDDEDDEE EDDEDDDEEE EADQGKKSKK
351 KSSAGHKKAG RSQLAEGQAG ERPPECKQQ
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K112	GAAEE **VKSE** QGEDK	0.93
2	K170	EEPKG **FKLE** FFFDQ	0.85
3	K71	YDEME **AKFF** EERAA	0.44
4	K226	ILKKK **PKKG** SKNTK	0.43
5	K223	TQKIL **KKKP** KKGSK	0.37
6	K358	SSAGH **KKAG** RSQLA	0.31
7	K167	SRVEE **PKGF** KLEFF	0.26

gene: At1g51950 **protein: IAA 18**

```
  1 MEGYSRNGEI SPKLLDLMIP QERRNWFHDE KNSVFKTEEK KLELKLGPPG
 51 EEDDDESMIR HMKKEPKDKS ILSLAGKYFS PSSTKTTSHK RTAPGPVVGW
101 PPVRSFRKNL ASGSSSKLGN DSTTSNGVTL KNQKCDAAAK TTEPKRQGGM
151 FVKINMYGVP IGRKVDLSAH NSYEQLSFTV DKLFRGLLAA QRDFPSSIED
201 EKPITGLLDG NGEYTLTYED NEGDKMLVGD VPWQMFVSSV KRLRVIKTSE
251 ISSALTYGNG KQEKMRR
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K36	EKNSV **FKTE** EKKLE	0.85
2	K63	SMIRH **MKKE** PKDKS	0.80
3	K45	EKKLE **LKLG** PPGEE	0.73
4	K261	LTYGN **GKQE** KMRR	0.67
5	K134	VTLKN **QKCD** AAAKT	0.50
6	K41	FKTEE **KKLE** LKLGP	0.48
7	K164	GVPIG **RKVD** LSAHN	0.44
8	K64	MIRHM **KKEP** KDKSI	0.37
9	K77	ILSLA **GKYF** SPSST	0.32
10	K182	LSFTV **DKLF** RGLLA	0.15

gene: At5g15840 **protein: CO**

```
  1 MLKQESNDIG SGENNRARPC DTCRSNACTV YCHADSAYLC MSCDAQVHSA
 51 NRVASRHKRV RVCESCERAP AAFLCEADDA SLCTACDSEV HSANPLARRH
101 QRVPILPISG NSFSSMTTTH HQSEKTMTDP EKRLVVDQEE GEEGDKDAKE
151 VASWLFPNSD KNNNNQNNGL LFSDEYLNLV DYNSSMDYKF TGEYSQHQQN
201 CSVPQTSYGG DRVVPLKLEE SRGHQCHNQQ NFQFNIKYGS SGTHYNDNGS
251 INHNAYISSM ETGVVPESTA CVTTASHPRT PKGTVEQQPD PASQMITVTQ
301 LSPMDREARV LRYREKRKTR KFEKTIRYAS RKAYAEIRPR VNGRFAKREI
351 EAEEQGFNTM LMYNTGYGIV PSF
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K3	M **LKQE** SNDIG	0.91
2	K217	DRVVP **LKLE** ESRGH	0.91
3	K347	VNGRF **AKRE** IEAEE	0.79
4	K237	NFQFN **IKYG** SSGTH	0.77
5	K321	EKRKT **RKFE** KTIRY	0.44

gene: At5g12840 **protein: Hap2a**

```
  1 MQSKPGRENE EEVNNHHAVQ QPMMYAEPWW KNNSFGVVPQ ARPSGIPSNS
 51 SSLDCPNGSE SNDVHSASED GALNGENDGT WKDSQAATSS RSVDNHGMEG
101 NDPALSIRNM HDQPLVQPPE LVGHYIACVP NPYQDPYYGG LMGAYGHQQL
151 GFRPYLGMPR ERTALPLDMA QEPVYVNAKQ YEGILRRRKA RAKAELERKV
201 IRDRKPYLHE SRHKHAMRRA RASGGRFAKK SEVEAGEDAG GRDRERGSAT
251 NSSGSEQVET DSNETLNSSG AP
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K193	RRKAR **AKAE** LERKV	0.79
2	K230	GGRFA **KKSE** VEAGE	0.48

gene: At2g22540 **protein: SVP**

```
  1 MAREKIQIRK IDNATARQVT FSKRRRGLFK KAEELSVLCD ADVALIIFSS
 51 TGKLFEFCSS SMKEVLERHN LQSKNLEKLD QPSLELQLVE NSDHARMSKE
101 IADKSHRLRQ MRGEELQGLD IEELQQLEKA LETGLTRVIE TKSDKIMSEI
151 SELQKKGMQL MDENKRLRQQ GTQLTEENER LGMQICNNVH AHGGAESENA
201 AVYEEGQSSE SITNAGNSTG APVDSESSDT SLRLGLPYGG
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K78	QSKNL **EKLD** QPSLE	0.50
2	K31	RRGLF **KKAE** ELSVL	0.48
3	K10	EKIQI **RKID** NATAR	0.44
4	K155	EISEL **QKKG** MQLMD	0.33
5	K53	IFSST **GKLF** EFCSS	0.32

gene: At5g10140 **protein: FLC**

```
  1 MGRKKLEIKR IENKSSRQVT FSKRRNGLIE KARQLSVLCD ASVALLVVSA
 51 SGKLYSFSSG DNLVKILDRY GKQHADDLKA LDHQSKALNY GSHYELLELV
101 DSKLVGSNVK NVSIDALVQL EEHLETALSV TRAKKTELML KLVENLKEKE
151 KMLKEENQVL ASQMENNHHV GAEAEMEMSP AGQISDNLPV TLPLLN
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K154	EKEKM **LKEE** NQVLA	0.91
2	K135	SVTRA **KKTE** LMLKL	0.48
3	K5	MGR **KKLE** IKRIE	0.48

gene: At2g32950 **protein: COP1**

```
  1 MEEISTDPVV PAVKPDPRTS SVGEGANRHE NDDGGSGGSE IGAPDLDKDL
 51 LCPICMQIIK DAFLTACGHS FCYMCIITHL RNKSDCPCCS QHLTNNQLYP
101 NFLLDKLLKK TSARHVSKTA SPLDQFREAL QRGCDVSIKE VDNLLTLLAE
151 RKRKMEQEEA ERNMQILLDF LHCLRKQKVD ELNEVQTDLQ YIKEDINAVE
201 RHRIDLYRAR DRYSVKLRML GDDPSTRNAW PHEKNQIGFN SNSLSIRGGN
251 FVGNYQNKKV EGKAQGSSHG LPKKDALSGS DSQSLNQSTV SMARKKRIHA
301 QFNDLQECYL QKRRQLADQP NSKQENDKSV VRREGYSNGL ADFQSVLTTF
351 TRYSRLRVIA EIRHGDIFHS ANIVSSIEFD RDDELFATAG VSRCIKVFDF
401 SSVVNEPADM QCPIVEMSTR SKLSCLSWNK HEKNHIASSD YEGIVTVWDV
451 TTRQSLMEYE EHEKRAWSVD FSRTEPSMLV SGSDDCKVKV WCTRQEASVI
501 NIDMKANICC VKYNPGSSNY IAVGSADHHI HYYDLRNISQ PLHVFSGHKK
551 AVSYVKFLSN NELASASTDS TLRLWDVKDN LPVRTFRGHT NEKNFVGLTV
601 NSEYLACGSE TNEVYVYHKE ITRPVTSHRF GSPDMDDAEE EAGSYFISAV
651 CWKSDSPTML TANSQGTIKV LVLAA
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K193	TDLQY **IKED** INAVE	0.94
2	K14	PVVPA **VKPD** PRTSS	0.93
3	K653	ISAVC **WKSD** SPTML	0.64
4	K273	SSHGL **PKKD** ALSGS	0.61
5	K396	GVSRC **IKVF** DFSSV	0.59
6	K178	HCLRK **QKVD** ELNEV	0.50
7	K259	GNYQN **KKVE** GKAQG	0.48
8	K154	LAERK **RKME** QEEAE	0.44
9	K593	RGHTN **EKNF** VGLTV	0.15

gene: At5g50870 **protein: UBC27**

```
  1 MIDFSRIQKE LQDCERNQDS SGIRVCPKSD NLTRLTGTIP GPIGTPYEGG
 51 TFQIDITMPD GYPFEPPKMQ FSTKVWHPNI SSQSGAICLD ILKDQWSPAL
101 TLKTALVSIQ ALLSAPEPKD PQDAVVAEQY MKNYQVFVST ARYWTETFAK
151 KSSLEEKVKR LVEMGFGDAQ VRSAIESSGG DENLALEKLC SA
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K28	GIRVC **PKSD** NLTRL	0.61
2	K119	LSAPE **PKDP** QDAVV	0.50

gene: At3g15355 **protein: PFU1**

```
  1 MEPNVVEIAT PPAASCSRIR TPTKAETPEV IDVEEYDLQN GGVPNGNNVD
 51 YKNKGKAIDF DSMSYGDYGE EDEYAVGSPG DDYGYPESSP LSNSLLDPES
101 LIYEDDENYS EQYDFEMEAE PDNYSMYQDL FDGKDIPTGV EVSMDWFPNS
151 ADKESASSSK SSHANNGNNS SKKATKASGI HSQFSSDMET PVAQPWNALP
201 HKAEGVIPNS AYALPQNSKA FQPPYAVHYS ALKTAFSNYL QPQTPDTVLG
251 EAPAPAAGSS GLLPPNTPGF KSNAARFKEE PPILPPDDSR VKRNMEDYLG
301 LYLFFKRFDI VEDFSDHHYA SKGTTSKQHS KDWAKRIQDE WRILEKDLPE
351 MIFVRAYESR MDLLRAVIIG AQGTPYHDGL FFFDIFFPDT YPSTPPIVHY
401 HSGGLRINPN LYNCGKVCLS LLGTWSGNQR EKWIPNTSTM LQVLVSIQGL
451 ILNQKPYFNE PGYERSAGSA HGESTSKAYS ENTFILSLKT MVYTMRRPPK
501 YFEDFAYGHF FSCAHDVLKA CNAYRNGATP GYLVKGAPDV EENSAGMSSL
551 KFRTDVATFV ETVLLKEFIL LGVLGLEPEE EEKTPETIIV AESSKCTRSR
601 SKRDRVSSS
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K278	SNAAR **FKEE** PPILP	0.85
2	K566	VETVL **LKEF** ILLGV	0.56
3	K202	WNALP **HKAE** GVIPN	0.52
4	K306	GLYLF **FKRF** DIVED	0.50
5	K583	EPEEE **EKTP** ETIIV	0.39
6	K500	TMRRP **PKYF** EDFAY	0.26

gene: At1g75940 **protein: β glucosidase**

```
  1 MGRFHKFPLL GLVLFLGLTG SLIAANEYAC SSTDIHFTRA NFPKGFIFGT
 51 ATAAFQVEGA VNEGCRGPSM WDVYTKKFPH KCNYHNADVA VDFYHRYKED
101 IKLMKNLNTD GFRFSIAWPR IFPHGRMEKG ISKAGVQYYH DLIDELLANG
151 ITPLVTVFHW DTPQDLEDEY GGFLSDRIIK DFTEYANFTF QEYGDKVKHW
201 ITFNEPWVFS RAGYDIGNKA PGRCSKYIKE HGEMCHDGRS GHEAYIVSHN
251 MLLAHADAVD AFRKCDKCKG GKIGIAHSPA WFEAHELSDE EHETPVTGLI
301 DFILGWHLHP TTYGDYPQSM KDHIGHRLPK FTEAQKEKLK NSADFVGINY
351 YTSVFALHDE EPDPSQPSWQ SDSLVDWEPR YVDKFNAFAN KPDVAKVEVY
401 AKGLRSLLKY IKDKYGNPEI MITENGYGED LGEQDTSLVV ALSDQHRTYY
451 IQKHLLSLHE AICDDKVNVT GYFHWSLMDN FEWQDGYKAR FGLYYVDYKN
501 NLTRHEKLSA QWYSSFLHDG SKEFEIEHEF EHDEL
```

SUMOylation consensus motifs (http://www.abgent.com/tools/sumoplot):

No.	Pos.	Group	Score
1	K396	NKPDV **AKVE** VYAKG	0.79
2	K180	LSDRI **IKDF** TEYAN	0.59
3	K272	DKCKG **GKIG** IAHSP	0.50
4	K264	AVDAF **RKCD** KCKGG	0.44
5	K6	MGRF **HKFP** LLGLV	0.41
6	K77	WDVYT **KKFP** HKCNY	0.37
7	K414	LKYIK **DKYG** NPEIM	0.33
8	K44	TRANF **PKGF** IFGTA	0.26

8.2 Analysis of flowering time of SUMO ligase mutants

8.2.1 Data of flowering time experiments of *pil1pil2* mutants

Below, the original data for the flowering time experiments concerning the SUMO ligase mutants are listed in Table 8.2.

Table 8.2: Data of flowering time analysis of different SUMO ligase mutants

☐ **LD**

date of sowing: 03.08.06

condition: LD 16 hours light per day

	Col WT			***siz1a***			***siz1c***			***pil1apil2b***			***pil1cpil2a***		
		leaves			**leaves**			**leaves**			**leaves**			**leaves**	
#	**date**	**r**	**c**	**date**	**r**	**c**	**date**	**r**	**c**	**date**	**r**	**c**	**date**	**r**	**c**
1	28.08.	13	1	05.09.	9	2	31.08.	10	2	31.08.	10	2	30.08.	11	2
2	30.08.	11	2	11.09.	11	2	04.09.	9	2	30.08.	12	2	30.08.	12	2
3	30.08.	15	3	11.09.	9	2	04.09.	9	2	30.08.	11	3	30.08.	11	2
4	28.08.	14	1	07.09.	10	0	04.09.	8	2	29.08.	10	2	31.08.	11	3
5	30.08.	11	3	08.09.	13	0	04.09.	11	3	29.08.	9	2	31.08.	11	2
6	30.08.	11	1	12.09.	12	2	05.09.	12	1	31.08.	13	2	30.08.	12	2
7	29.08.	12	1	11.09.	11	2	11.09.	8	2	30.08.	8	3	31.08.	10	2
8	28.08.	11	1	19.09.	14	2	05.09.	9	2	30.08.	11	3	04.09.	10	2
9	29.08.	11	1	07.09.	11	3	06.09.	8	1	29.08.	11	2	30.08.	8	2
10	28.08.	10	1				05.09.	11	2	29.08.	10	2	30.08.	12	3
11	29.08.	10	3				08.09.	11	2	29.08.	11	2	31.08.	12	2
12	29.08.	9	3				06.09.	10	1	30.08.	11	3	29.08.	9	1
13	28.08.	12	1				05.09.	7	1	30.08.	8	2	31.08.	11	2
14	31.08.	11	2				06.09.	8	1	31.08.	14	2	30.08.	10	1
15	29.08.	10	1				06.09.	9	2	30.08.	14	1	04.09.	15	2
16	28.08.	10	1				08.09.	10	2	28.08.	12	2	30.08.	10	2
17	28.08.	11	1				06.09.	9	2	29.08.	11	2	29.08.	10	2
18	28.08.	11	1				06.09.	9	1	29.08.	12	2	30.08.	11	3
19	29.08.	10	2							31.08.	10	2	04.09.	13	2
20	29.08.	13	2							29.08.	11	3	28.08.	9	2
21	28.08.	11	2										04.09.	13	2

LD

date of sowing: 29.09.06

condition: LD 16 hours light per day

	Col WT			*siz1a*			*siz1c*			*siz1f*			*pil1apil2b*			*pil1cpil2a*		
		leaves			leaves			leaves			leaves			leaves			leaves	
#	date	r	c	date	r	c	date	r	c	date	r	c	date	r	c	date	r	c
1	07.11.	39	4	07.11.	6	4	02.11.	12	2	06.11.	29	4	07.11.	28	4	04.11.	31	5
2	06.11.	36	4	08.11.	7	2	31.10.	14	3	07.11.	18	3	06.11.	37	4	08.11.	30	4
3	06.11.	28	3	08.11.	6	4	31.10.	15	4	08.11.	22	3	06.11.	26	4	10.11.	32	5
4	04.11.	34	5	09.11.	7	4	09.11.	6	2	07.11.	28	4	06.11.	26	3	06.11.	22	4
5	06.11.	25	4	06.11.	12	3	06.11.	5	3	06.11.	13	4	04.11.	26	3	07.11.	25	5
6	30.10.	25	4	06.11.	12	3	09.11.	8	3	09.11.	20	4	07.11.	28	5	08.11.	19	3
7	04.11.	32	5	06.11.	7	4	06.11.	5	3	02.11.	24	4	06.11.	29	4	30.10.	18	4
8	27.10.	9	2	31.10.	7	4	06.11.	5	4	31.10.	24	3	29.10.	16	3	30.10.	15	3
9	29.10.	19	2	06.11.	7	3	30.10.	19	3	02.11.	21	4	31.10.	18	4	30.10.	16	4
10	27.10.	16	2	06.11.	7	5	31.10.	17	4	03.11.	20	6	31.10.	25	4	02.11.	22	3
11	06.11.	28	5	03.11.	14	3	31.10.	11	3	03.11.	13	4	31.10.	16	3	31.10.	19	3
12	07.11.	39	6	04.11.	10	3	03.11.	6	3	04.11.	13	4	08.11.	24	5	07.11.	39	6
13	08.11.	49	4	31.10.	13	3	08.11.	5	2	04.11.	21	4	06.11.	22	4	08.11.	27	5
14	31.10.	21	4	31.10.	16	2	04.11.	9	4	31.10.	21	3	06.11.	41	5	07.11.	29	5
15				31.10.	10	3	04.11.	10	3	06.11.	14	5	02.11.	17	4	06.11.	24	4
16				31.10.	9	3	04.11.	15	4	06.11.	28	4	04.11.	32	6	06.11.	37	5
17				02.11.	16	3	29.10.	8	2	08.11.	27	5	04.11.	27	6	06.11.	34	4

extended SD

date of sowing: 03.08.06

condition: LD 16 hours light per day

	Col WT			*siz1a*			*siz1c*			*pil1apil2b*			*pil1cpil2a*		
		leaves			leaves			leaves			leaves			leaves	
#	date	r	c	date	r	c	date	r	c	date	r	c	date	r	c
1	19.09.	46	7	19.09.	27	6	19.09.	26	6	19.09.	36	5	19.09.	43	6
2	19.09.	47	7	13.09.	18	2	21.09.	46	5	14.09.	38	5	19.09.	48	6
3	19.09.	48	7	19.09.	36	8	19.09.	35	5	14.09.	29	6	19.09.	40	5
4	21.09.	44	6	13.09.	21	3	19.09.	30	6	19.09.	47	5	15.09.	43	5
5	19.09.	41	4	19.09.	35	5	19.09.	25	5	15.09.	31	6	19.09.	39	6
6	19.09.	46	5	22.09.	31	5	13.09.	18	1	15.09.	33	6	19.09.	40	6
7	19.09.	40	7	11.09.	18	0	19.09.	31	5	15.09.	41	6	19.09.	40	6
8	19.09.	43	8	19.09.	26	4	19.09.	35	2	15.09.	33	6	19.09.	43	7
9	15.09.	43	6	19.09.	29	4	19.09.	24	5	14.09.	28	5	19.09.	48	6
10	13.09.	36	5	19.09.	20	1	19.09.	30	5	14.09.	31	5	19.09.	49	6
11	19.09.	47	5	15.09.	26	5	15.09.	27	5	19.09.	38	7	19.09.	50	6
12	13.09.	41	5	19.09.	23	3	13.09.	20	3	19.09.	47	6	19.09.	44	6
13	15.09.	39	6	19.09.	23	3	19.09.	30	4	19.09.	38	7	19.09.	54	7
14	19.09.	35	7	19.09.	31	5	19.09.	39	4	19.09.	40	5	19.09.	53	8
15	19.09.	35	5	14.09.	18	4	19.09.	30	4	19.09.	38	6	20.09.	50	6
16	19.09.	40	6	19.09.	24	5	19.09.	32	4	19.09.	35	9	19.09.	44	6
17	19.09.	55	6	20.09.	37	6	21.09.	47	5	19.09.	41	6	19.09.	50	6
18	15.09.	37	6	19.09.	30	6	19.09.	35	4	15.09.	41	7	19.09.	40	6
19	20.09.	47	5	19.09.	41	6	19.09.	35	5	19.09.	35	6	20.09.	51	7
20	19.09.	37	7	19.09.	27	5	19.09.	28	3	19.09.	32	6	19.09.	45	6
21	19.09.	42	6							15.09.	35	4	19.09.	42	6

SD

date of sowing: 29.09.06

condition: SD 8 hours light per day

	Col WT			*siz1a*			*siz1c*			*siz1f*			*pil1apil2b*			*pil1cpil2a*		
		leaves			leaves			leaves			leaves			leaves			leaves	
#	date	r	c	date	r	c	date	r	c	date	r	c	date	r	c	date	r	c
1	04.12.	89	9	29.12.	55	7	03.01.	76	5	19.12.	96	10	04.12.	101	8	01.12.	82	9
2	05.12.	85	8	26.12.	43	4	12.01.	40	6	12.12.	88	12	12.12.	111	8	01.12.	109	9
3	04.12.	86	10	11.12.	15	6	04.12.	57	5	15.12.	89	11	18.12.	77	11	06.12.	100	10
4	12.11.	37	5	09.01.	94	6	12.12.	58	6	13.12.	89	11	11.12.	70	10	06.12.	109	11
5	12.11.	38	5	15.12.	37	4	29.12.	48	3	22.12.	94	11	05.12.	82	9	04.12.	109	8
6	08.12.	85	7	01.01.	65	7	22.01.	100	4	29.12.	70	4	04.12.	81	8	01.12.	78	9
7	14.11.	49	5	12.12.	34	3	11.01.	45	4	11.12.	78	11	06.12.	90	8	07.12.	96	8
8	15.11.	38	5	26.12.	43	5	22.01.	99	4	01.01.	106	11	01.12.	95	8	09.12.	96	9
9	05.12.	82	8	20.12.	60	7	04.01.	56	6	11.12.	83	7	08.12.	93	9	30.11.	91	8
10	05.12.	79	9	11.01.	87	7	26.12.	66	5	14.12.	95	8	04.12.	70	8	04.12.	103	8
11	09.11.	34	5	01.01.	71	8	01.12.	65	5	18.12.	117	10	04.12.	73	9	01.12.	87	9
12	11.12.	88	10	14.12.	60	5	22.12.	58	6	04.01.	94	5	01.12.	81	8	04.12.	81	8
13	13.11.	43	5	30.11.	41	6	13.12.	87	8	29.12.	91	9	08.12.	86	10	04.12.	72	8
14	18.11.	51	3	22.12.	51	3	09.01.	64	6	01.01.	104	11	04.12.	89	9	14.12.	101	8
15				12.12.	31	6	13.12.	32	6	10.12.	100	10	30.11.	92	7	11.12.	80	10
16							11.12.	40	6	11.12.	79	10	28.11.	94	8	15.12.	98	8
17													28.11.	93	8	04.12.	75	10

The original data for the flowering time determination of the different SUMO ligase mutants are shown above. Rosette leaves (r) and cauline leaves (c) were counted at the opening of the first flower bud.

8.2.2 Data of flowering time experiments of *el1* mutants

☐ **LD**

date of sowing: 10.03.08

condition: LD 16 hours light per day

	Col WT			WS WT			*el1a*			*el1b*			*el1c*			*siz1f*		
		leaves			leaves			leaves			leaves			leaves			leaves	
#	date	r	c	date	r	c	date	R	c	date	r	c	date	r	c	date	r	c
1	10.04.	13	3	02.04.	6	2	07.04.	10	7	12.04.	12	3	09.04.	12	3	10.04.	11	4
2	13.04.	10	3	05.04.	11	3	12.04.	9	9	14.04.	15	4	12.04.	13	3	10.04.	10	4
3	09.04.	11	3	07.04.	8	3	07.04.	10	6	10.04.	10	3	12.04.	14	3	12.04.	9	2
4	12.04.	13	2	05.04.	9	3	08.04.	10	7	12.04.	14	3	09.04.	9	3	10.04.	11	3
5	14.04.	13	3	07.04.	9	3	05.04.	10	6	12.04.	12	2	09.04.	8	3	08.04.	11	3
6	10.04.	12	3	04.04.	9	2	12.04.	10	6	08.04.	12	2	07.04.	11	3	08.04.	11	2
7	08.04.	14	2	03.04.	9	3	07.04.	10	6	08.04.	11	2	08.04.	13	3	10.04.	11	2
8	10.04.	11	3	03.04.	8	3	05.04.	9	8	07.04.	14	3	12.04.	11	2	10.04.	8	3
9	15.04.	15	3	07.04.	13	3	10.04.	11	6	12.04.	13	2	10.04.	10	2	08.04.	10	3
10	12.04.	10	2	05.04.	9	3	07.04.	8	5	09.04.	13	3	12.04.	12	2	07.04.	11	2
11	13.04.	12	2	05.04.	6	5	12.04.	9	6	12.04.	13	2	09.04.	9	3	09.04.	9	3
12							07.04.	9	6	10.04.	9	2						

☐ **LD**

date of sowing: 05.05.08

condition: LD 16 hours light per day

	Col WT			WS WT			*el1a*			*el1b*			*el1c*			*siz1f*		
		leaves			leaves			leaves			leaves			leaves			leaves	
#	date	r	c	date	r	c	date	R	c	date	r	c	date	r	c	date	r	c
1	07.06.	27	2	05.06.	10	3	05.06.	9	2	10.06.	17	3	10.06.	19	4	08.06.	13	4
2	10.06.	16	4	05.06.	10	3	07.06.	11	3	12.06.	17	4	11.06.	19	4	07.06.	15	4
3	05.06.	15	3	05.06.	12	3	05.06.	10	3	12.06.	22	4	12.06.	14	3	08.06.	17	4
4	09.06.	14	3	05.06.	8	4	05.06.	11	3	07.06.	17	3	08.06.	20	4	07.06.	11	4
5	07.06.	13	3	05.06.	8	3	07.06.	11	3	13.06.	18	4	13.06.	13	4	07.06.	21	3
6	10.06.	13	3	05.06.	8	4	07.06.	11	3	12.06.	15	4	11.06.	20	5	05.06.	11	3
7	09.06.	13	3	05.06.	9	3	07.06.	12	3	08.06.	12	3	10.06.	14	4	07.06.	10	2
8	07.06.	11	2	05.06.	9	3	05.06.	12	2	08.06.	18	3	05.06.	13	3	08.06.	15	3
9	05.06.	12	3	07.06.	11	3	05.06.	10	2	10.06.	13	4	06.06.	13	3	07.06.	9	3
10	07.06.	13	3	05.06.	11	4	05.06.	11	2	13.06.	11	3	13.06.	15	3	06.06.	11	3
11	08.06.	11	2				05.06.	11	3	06.06.	12	3	08.06.	13	3	07.06.	14	3
12							05.06.	11	3	07.06.	18	4	11.06.	11	2	07.06.	12	3

SD

date of sowing: 05.05.08

condition: SD 8 hours light per day

	Col WT			WS WT			*el1a*			*el1b*			*el1c*			*siz1f*		
		leaves			leaves			leaves			leaves			leaves			leaves	
#	date	r	c	date	r	c	date	R	c	date	r	c	date	r	c	date	r	c
1	28.07.	99	9	11.07.	71	8	13.07.	63	7	05.07.	105	9	30.07.	113	7	07.08.	64	8
2	21.07.	92	6	05.07.	63	6	17.07.	20	5	28.07.	77	10	11.08.	83	8	29.07.	63	8
3	14.07.	55	15	28.07.	84	9	13.07.	83	7	11.08.	100	9	19.07.	105	9	18.07.	22	11
4	05.07.	108	9	18.07.	69	8	13.07.	68	8	13.07.	65	15	30.07.	86	8	18.07.	31	12
5	08.07.	52	11	15.07.	86	7	10.07.	60	7	08.07.	56	11	15.07.	56	14	19.07.	28	10
6	07.07.	29	12	18.07.	96	7	10.07.	36	6	14.07.	49	16	14.07.	82	16	17.07.	41	15
7	09.07.	73	13	27.06.	111	8	07.07.	20	4	30.07.	31	2	07.07.	99	11	13.07.	65	12
8	09.07.	69	12	17.07.	12	7	07.07.	45	7	17.07.	33	12	13.07.	56	15	14.07.	46	10
9				13.07.	18	8	30.06.	82	3	13.07.	63	15	17.07.	42	13	13.07.	36	10
10				07.07.	37	8	09.07.	16	4				09.07.	66	12			
11				08.07.	46	8	07.07.	45	6									

8.3 Data analysis with SigmaPlot 10 and SigmaStat3

In the following, the data analysis of the flowering time experiments for the *siz1* and *pil* mutants is shown that was conducted with SigmaPlot 10 and SigmaStat3.

Table 8.3: Data of flowering time analysis of different SUMO ligase mutants

☐ **LD**

One Way Analysis of Variance

Dependent Variable: Rosette leaves

Normality Test: Failed (P < 0.050) **Equal Variance Test:** Passed (P = 0.471)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Col WT	21	0	11.286	1.454	0.317
siz1a	9	0	11.111	1.691	0.564
siz1c	18	0	9.333	1.328	0.313
pil1apil2b	20	0	10.950	1.638	0.366
pil1cpil2a	21	0	11.000	1.581	0.345
Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	4	45.403	11.351	4.862	0.001
Residual	84	196.125	2.335		
Total	88	241.528			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001).
Power of performed test with alpha = 0.050: 0.890
All Pairwise Multiple Comparison Procedures (Holm-Sidak method): Overall significance level = 0.01
Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**T**	**Unadjusted P**	**Critical Level**	**Significant?**
Col WT vs. *siz1c*	1.952	3.978	0.000147	0.001	Yes
pil1cpil2a vs. *siz1c*	1.667	3.396	0.00105	0.001	Yes
pil1apil2b vs. *siz1c*	1.617	3.257	0.00163	0.001	No
siz1a vs. *siz1c*	1.778	2.850	0.00550	0.001	No
Col WT vs. *pil1apil2b*	0.336	0.703	0.484	0.002	No
Col WT vs. *pil1cpil2a*	0.286	0.606	0.546	0.002	No
Col WT vs. *siz1a*	0.175	0.287	0.775	0.003	No
siz1a vs. *pil1apil2b*	0.161	0.263	0.793	0.003	No
siz1a vs. *pil1cpil2a*	0.111	0.183	0.856	0.005	No
pil1cpil2a vs. *pil1apil2b*	0.0500	0.105	0.917	0.010	No

☐ **LD**

One Way Analysis of Variance

Dependent Variable: Cauline leaves

Normality Test: Failed (P < 0.050) **Equal Variance Test:** Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
Col WT	21	0	1.619	0.805	0.176
siz1a	9	0	1.667	1.000	0.333
siz1c	18	0	1.722	0.575	0.135
pil1apil2b	20	0	2.200	0.523	0.117
pil1cpil2a	21	0	2.048	0.498	0.109

Source of Variation	DF	SS	MS	F	P
Between Groups	4	4.925	1.231	2.817	0.030
Residual	84	36.716	0.437		
Total	88	41.640			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.030).

Power of performed test with alpha = 0.050: 0.525

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	Diff of Means	T	Unadjusted P	Critical Level	Significant?
pil1apil2b vs. Col WT	0.581	2.812	0.00612	0.001	No
pil1apil2b vs. *siz1c*	0.478	2.224	0.0288	0.001	No
pil1cpil2a vs. Col WT	0.429	2.101	0.0387	0.001	No
pil1apil2b vs. *siz1a*	0.533	2.010	0.0477	0.001	No
pil1cpil2a vs. *siz1c*	0.325	1.532	0.129	0.002	No
pil1cpil2a vs. *siz1a*	0.381	1.446	0.152	0.002	No
pil1apil2b vs. *pil1cpil2a*	0.152	0.738	0.463	0.003	No
siz1c vs. Col WT	0.103	0.486	0.628	0.003	No
siz1c vs. *siz1a*	0.0556	0.206	0.837	0.005	No
siz1a vs. Col WT	0.0476	0.181	0.857	0.010	No

☐ **LD**

One Way Analysis of Variance

Dependent Variable: Rosette leaves

Normality Test: Passed (P = 0.235) **Equal Variance Test:** Failed (P < 0.050)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Col WT	14	0	28.571	10.552	2.820
siz1a	17	0	9.765	3.456	0.838
siz1c	17	0	10.000	4.623	1.121
siz1f	17	0	20.941	5.414	1.313
pil1apil2b	17	0	25.765	6.915	1.677
pil1cpil2a	17	0	25.824	7.333	1.779
Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	5	5664.820	1132.964	25.857	<0.001
Residual	93	4074.958	43.817		
Total	98	9739.778			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**T**	**Unadjusted P**	**Critical Level**	**Significant?**
Col WT vs. *siz1a*	18.807	7.872	6.259E-012	0.001	Yes
Col WT vs. *siz1c*	18.571	7.774	1.003E-011	0.001	Yes
pil1cpil2a vs. *siz1a*	16.059	7.073	0.000000000278	0.001	Yes
pil1apil2b vs. *siz1a*	16.000	7.047	0.000000000314	0.001	Yes
pil1cpil2a vs. *siz1c*	15.824	6.969	0.000000000451	0.001	Yes
pil1apil2b vs. *siz1c*	15.765	6.943	0.000000000509	0.001	Yes
siz1f vs. *siz1a*	11.176	4.923	0.00000369	0.001	Yes
siz1f vs. *siz1c*	10.941	4.819	0.00000560	0.001	Yes
Col WT vs. *siz1f*	7.630	3.194	0.00192	0.001	No
pil1cpil2a vs. *siz1f*	4.882	2.150	0.0341	0.002	No
pil1apil2b vs. *siz1f*	4.824	2.124	0.0363	0.002	No
Col WT vs. *pil1apil2b*	2.807	1.175	0.243	0.003	No
Col WT vs. *pil1cpil2a*	2.748	1.150	0.253	0.003	No
siz1c vs. *siz1a*	0.235	0.104	0.918	0.005	No
pil1cpil2a vs. *pil1apil2b*	0.0588	0.0259	0.979	0.010	No

☐ **LD**

One Way Analysis of Variance

Dependent Variable: Cauline leaves

Normality Test: Failed (P < 0.050) **Equal Variance Test:** Passed (P = 0.627)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Col WT	14	0	3.857	1.231	0.329
siz1a	17	0	3.294	0.772	0.187
siz1c	17	0	3.059	0.748	0.181
siz1f	17	0	4.000	0.791	0.192
pil1apil2b	17	0	4.176	0.951	0.231
pil1cpil2a	17	0	4.235	0.903	0.219

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	5	19.942	3.988	4.899	<0.001
Residual	93	75.714	0.814		
Total	98	95.657			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.940

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**T**	**Unadjusted P**	**Critical Level**	**Significant?**
pil1cpil2a vs. *siz1c*	1.176	3.801	0.000257	0.001	Yes
pil1apil2b vs. *siz1c*	1.118	3.611	0.000494	0.001	Yes
siz1f vs. *siz1c*	0.941	3.041	0.00306	0.001	No
pil1cpil2a vs. *siz1a*	0.941	3.041	0.00306	0.001	No
pil1apil2b vs. *siz1a*	0.882	2.851	0.00537	0.001	No
Col WT vs. *siz1c*	0.798	2.452	0.0161	0.001	No
siz1f vs. *siz1a*	0.706	2.281	0.0248	0.001	No
Col WT vs. *siz1a*	0.563	1.729	0.0871	0.001	No
pil1cpil2a vs. Col WT	0.378	1.161	0.249	0.001	No
pil1apil2b vs. Col WT	0.319	0.981	0.329	0.002	No
pil1cpil2a vs. *siz1f*	0.235	0.760	0.449	0.002	No
siz1a vs. *siz1c*	0.235	0.760	0.449	0.003	No
pil1apil2b vs. siz1f	0.176	0.570	0.570	0.003	No
siz1f vs. Col WT	0.143	0.439	0.662	0.005	No
pil1cpil2a vs. *pil1apil2b*	0.0588	0.190	0.850	0.010	No

extended SD

One Way Analysis of Variance

Dependent Variable: Rosette leaves

Normality Test: Passed (P = 0.055) **Equal Variance Test:** Passed (P = 0.289)

Group Name	N	Missing	Mean	Std Dev	SEM
Col WT	21	0	42.333	5.092	1.111
siz1a	21	0	28.143	8.242	1.799
siz1c	21	0	30.857	7.330	1.600
pil1apil2b	21	0	36.524	5.212	1.137
pil1cpil2a	21	0	45.524	4.708	1.027

Source of Variation	DF	SS	MS	F	P
Between Groups	4	4556.705	1139.176	28.926	<0.001
Residual	100	3938.286	39.383		
Total	104	8494.990			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	Diff of Means	T	Unadjusted P	Critical Level	Significant?
pil1cpil2a vs. *siz1a*	17.381	8.975	1.746E-014	0.001	Yes
pil1cpil2a vs. *siz1c*	14.667	7.573	1.859E-011	0.001	Yes
Col WT vs. *siz1a*	14.190	7.327	6.161E-011	0.001	Yes
Col WT vs. *siz1c*	11.476	5.926	0.0000000444	0.001	Yes
pil1cpil2a vs. *pil1apil2b*	9.000	4.647	0.0000103	0.002	Yes
pil1apil2b vs. *siz1a*	8.381	4.327	0.0000358	0.002	Yes
Col WT vs. *pil1apil2b*	5.810	3.000	0.00341	0.003	No
pil1apil2b vs. *siz1c*	5.667	2.926	0.00425	0.003	No
pil1cpil2a vs. Col WT	3.190	1.647	0.103	0.005	No
siz1c vs. *siz1a*	2.714	1.402	0.164	0.010	No

extended SD

One Way Analysis of Variance

Dependent Variable: Cauline leaves

Normality Test: Failed (P < 0.050) **Equal Variance Test:** Failed (P < 0.050)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Col WT	21	0	6.000	1.000	0.218
siz1a	21	0	4.381	1.884	0.411
siz1c	21	0	4.333	1.238	0.270
pil1apil2b	21	0	5.905	1.044	0.228
pil1cpil2a	21	0	6.143	0.655	0.143
Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	4	69.962	17.490	11.507	<0.001
Residual	100	152.000	1.520		
Total	104	221.962			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**T**	**Unadjusted P**	**Critical Level**	**Significant?**
*pil1cpil2*a vs. *siz1c*	1.810	4.756	0.00000665	0.001	Yes
pil1cpil2a vs. *siz1a*	1.762	4.631	0.0000110	0.001	Yes
Col WT vs. *siz1c*	1.667	4.380	0.0000292	0.001	Yes
Col WT vs. *siz1a*	1.619	4.255	0.0000471	0.001	Yes
pil1apil2b vs. *siz1c*	1.571	4.130	0.0000753	0.002	Yes
pil1apil2b vs. *siz1a*	1.524	4.005	0.000119	0.002	Yes
pil1cpil2a vs. *pil1apil2b*	0.238	0.626	0.533	0.003	No
pil1cpil2a vs. Col WT	0.143	0.375	0.708	0.003	No
Col WT vs. *pil1apil2b*	0.0952	0.250	0.803	0.005	No
siz1a vs. *siz1c*	0.0476	0.125	0.901	0.010	No

SD

One Way Analysis of Variance

Dependent Variable: Rosette leaves

Normality Test: Passed (P = 0.636) **Equal Variance Test:** Failed (P < 0.050)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Col WT	14	0	63.143	23.075	6.167
siz1a	15	0	52.467	21.206	5.475
siz1c	16	0	61.938	20.181	5.045
siz1f	16	0	92.063	11.625	2.906
pil1apil2b	17	0	86.941	11.088	2.689
pil1cpil2a	17	0	92.176	12.441	3.017

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	5	24380.424	4876.085	16.823	<0.001
Residual	89	25796.734	289.851		
Total	94	50177.158			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
pil1cpil2a vs. *siz1a*	39.710	6.584	0.00000000309	0.001	Yes
siz1f vs. *siz1a*	39.596	6.471	0.00000000514	0.001	Yes
pil1apil2b vs. *siz1a*	34.475	5.716	0.000000143	0.001	Yes
pil1cpil2a vs. *siz1c*	30.239	5.099	0.00000191	0.001	Yes
siz1f vs. *siz1c*	30.125	5.005	0.00000280	0.001	Yes
pil1cpil2a vs. Col WT	29.034	4.725	0.00000854	0.001	Yes
siz1f vs. Col WT	28.920	4.642	0.0000118	0.001	Yes
pil1apil2b vs. siz1c	25.004	4.216	0.0000595	0.001	Yes
pil1apil2b vs. Col WT	23.798	3.873	0.000205	0.001	Yes
Col WT vs. *siz1a*	10.676	1.687	0.0950	0.002	No
siz1c vs. *siz1a*	9.471	1.548	0.125	0.002	No
pil1cpil2a vs. *pil1apil2b*	5.235	0.897	0.372	0.003	No
siz1f vs. *pil1apil2b*	5.121	0.864	0.390	0.003	No
Col WT vs. *siz1c*	1.205	0.193	0.847	0.005	No
pil1cpil2a vs. *siz1f*	0.114	0.0192	0.985	0.010	No

SD

One Way Analysis of Variance

Dependent Variable: Cauline leaves

Normality Test: Failed (P < 0.050)) **Equal Variance Test:** Passed (P = 0.121)

Group Name	N	Missing	Mean	Std Dev	SEM
Col WT	14	0	6.714	2.268	0.606
siz1a	15	0	5.600	1.549	0.400
siz1c	16	0	5.313	1.195	0.299
siz1f	16	0	9.438	2.308	0.577
pil1apil2b	17	0	8.588	1.004	0.243
pil1cpil2a	17	0	8.824	0.951	0.231

Source of Variation	DF	SS	MS	F	P
Between Groups	5	249.264	49.853	19.090	<0.001
Residual	89	232.420	2.611		
Total	94	481.684			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):Overall significance level = 0.01

Comparisons for factor: **Genotype**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
siz1f vs. *siz1c*	4.125	7.220	0.000000000168	0.001	Yes
siz1f vs. *siz1a*	3.838	6.607	0.00000000278	0.001	Yes
pil1cpil2a vs. *siz1c*	3.511	6.238	0.0000000146	0.001	Yes
pil1apil2b vs. *siz1c*	3.276	5.820	0.0000000916	0.001	Yes
pil1cpil2a vs. *siz1a*	3.224	5.631	0.000000206	0.001	Yes
pil1apil2b vs. *siz1a*	2.988	5.220	0.00000116	0.001	Yes
siz1f vs. Col WT	2.723	4.605	0.0000137	0.001	Yes
pil1cpil2a vs. Col WT	2.109	3.617	0.000495	0.001	Yes
pil1apil2b vs. Col WT	1.874	3.213	0.00183	0.001	No
Col WT vs. *siz1c*	1.402	2.370	0.0199	0.002	No
Col WT vs. *siz1a*	1.114	1.856	0.0668	0.002	No
siz1f vs. *pil1apil2b*	0.849	1.509	0.135	0.003	No
siz1f vs. *pil1cpil2a*	0.614	1.091	0.278	0.003	No
siz1a vs. *siz1c*	0.287	0.495	0.622	0.005	No
pil1cpil2a vs. *pil1apil2b*	0.235	0.425	0.672	0.010	No

Table 8.4: Data of flowering time analysis of *el1* mutants

☐ **LD**

One Way Analysis of Variance

Dependent Variable: Rosette leaves

Normality Test: Failed (P < 0.050) **Equal Variance Test:** Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
Col WT	11	0	12.182	1.601	0.483
WS WT	11	0	8.818	1.991	0.600
el1a	12	0	9.583	0.793	0.229
el1b	12	0	12.333	1.723	0.497
el1c	11	0	11.091	1.921	0.579
siz1f	11	0	10.182	1.079	0.325

Source of Variation	DF	SS	MS	F	P
Between Groups	5	114.716	22.943	9.273	<0.001
Residual	62	153.402	2.474		
Total	67	268.118			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 1.000
All Pairwise Multiple Comparison Procedures (Holm-Sidak method): Overall significance level = 0.01
Comparisons for factor: **Genotype**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
el1b vs. WS WT	3.515	5.354	0.00000133	0.001	Yes
Col WT vs. WS WT	3.364	5.015	0.00000472	0.001	Yes
el1b vs. *el1a*1	2.750	4.282	0.0000654	0.001	Yes
Col WT vs. *el1a*	2.598	3.958	0.000198	0.001	Yes
el1c vs. WS WT	2.273	3.389	0.00123	0.001	No
el1b vs. *siz1f*	2.152	3.277	0.00172	0.001	No
Col WT vs. *siz1f*	2.000	2.982	0.00409	0.001	No
el1c vs. *el1a*1	1.508	2.296	0.0251	0.001	No
siz1f vs. WS WT	1.364	2.033	0.0463	0.001	No
el1b vs. *el1c*3	1.242	1.892	0.0631	0.002	No
Col WT vs. *el1c*	1.091	1.626	0.109	0.002	No
el1c vs. siz1f	0.909	1.355	0.180	0.003	No
el11a vs. WS WT	0.765	1.165	0.248	0.003	No
siz1f vs. *el1a*	0.598	0.912	0.366	0.005	No
el1b vs. Col WT	0.152	0.231	0.818	0.010	No

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ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. George Coupland betreut worden.

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